A PHYSICAL, CULTURAL, AND NUTRITIONAL STUDY OF *LEPTOSPIRA INTERROGANS* SEROTYPE *HARDJO* WITH EMPHASIS ON ANTIGENICITY, IMMUNOGENICITY, AND VIABILITY

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An abstract of a Thesis by
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The problem. *Leptospira interrogans* serotype *hardjo* has been indicated as the cause of abortion and clinical leptospirosis in cattle. It is a fastidious organism and has been difficult to reisolate from infected animals. This study was designed to determine if the nutritional requirements were different from other leptospires and if an optimum incubation temperature could be established. Secondly, studies were done to see what effects these parameters had on antigenicity and immunogenicity.

Procedure. Cells of an avirulent laboratory adapted strain of *L. hardjo* were grown in different formulations of bovine albumin polysorbate media (BA-P80). Single and multiple deletions and different sources of ingredients for the medium were evaluated. Continuous growth was measured over a predetermined range of temperatures. The antigenicity of cells incubated at two temperatures were compared in a microscopic agglutination test. The efficacy of two heat-inactivated bacterins was evaluated by challenge of vaccinated hamsters with a kidney infective isolate.

Findings. The avirulent strain grew best at temperatures between 29° and 31.5° C. Optimum growth occurred in the complete BA-P80 media formulation. No difference was seen in the antigenicity of either 29° or 37° C incubated leptospires. The bacterin prepared with whole cells grown at 37° C was more efficacious than the bacterin using cells grown at 29° C.

Conclusions. The nutritional requirements for *L. hardjo* did not differ from many of the other leptospires that have been studied. Bovine albumin polysorbate 80 media was best suited for routine propagation of antigens and isolation of leptospires from infected tissues. Heat-inactivated whole cell bacterins provided excellent protection.

Recommendations. Attempts should be made to duplicate the nutritional studies and growth temperature studies using several virulent *L. hardjo* isolates. Further research needs to be done to determine why a whole cell bacterin prepared using antigen incubated at 37° C provided greater immunity.

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INTRODUCTION

Leptospira interrogans serotype *hardjo* strain *Hardjoprajitno* was isolated from the blood of a febrile Javanese plantation worker in 1938 (Wolff 1952).

The first isolate in the United States was recovered from a Louisiana cattle herd. A routine serological survey was conducted in order to determine the prevalence of *Leptospira pomona* infections, the primary etiologic agent of bovine leptospirosis. A low incidence of serum reactors was noted against the antigens of the hebdomadis serogroup. In order to better define the infective serotype, cross-agglutination studies with the new isolate against 17 members of the hebdomadis serogroup identified the isolate as *Leptospira hardjo* (Roth and Galton 1960). Prior to this report, no animal host either wild or domestic was known for the *L. hardjo* serotype.

A second report of *L. hardjo* infection occurred in a Pennsylvania cattle herd (Clark et al. 1961). Urine samples from a cow exhibiting clinical symptoms were inoculated into hamsters in an attempt to isolate a pure culture of the pathogenic agent. Leptospires were recovered in Chang's media which had been inoculated with hamster kidney material from the first hamster passage of bovine urine. Once again cross-agglutination serologic studies confirmed the spirochetes as *Leptospira hardjo*.

Robertson and coworkers (1964) described the first case of an actual abortion in cattle directly correlated to the

isolation of L. hardjo from the urine of the dams. Cattle exhibiting atypical mastitis were screened serologically in order to estimate the incidence of leptospira agglutinins in the herd. Serologic titers for L. pomona antigen were negative, however, when L. sejroe (a hebdomadis serogroup member) antigen was used, titers of 1:1000 to 1:10000 were recorded. Urine was collected from the ill animals and inoculated into guinea pigs. Nine serial passages of blood from guinea pigs were necessary before it was possible to isolate the organism in a modified Stuart's semisolid medium supplemented with guinea pig cardiac blood. Further in vitro passages were possible only when Stuart's medium containing rabbit serum was further enriched with guinea pig blood. The substitutions of hamster, calf, or increased amounts of rabbit blood were unsatisfactory. Fresh guinea pig blood could be replaced by 10 percent guinea pig serum with 10 percent hemoglobin. Further serological investigation identified the isolate as being homologous to Roth's LSU-91 isolate of Leptospira hardjo. Robertson was one of the first to propose that the low incidence of isolation of the hardjo serotype was due to its extreme fastidious nutritional requirements.

Additional evidence that L. hardjo infection may be a widespread cause of bovine infertility was reported in 1964 (Sulzer et al. 1964). A serologic survey of a herd with a history of recent abortions revealed agglutinin titers to

serotype L. sejroe in every member of the herd. Two animals also were reactors to L. pomona antigen. Leptospires were reportedly isolated directly from the urine of two animals even though they had been vaccinated with a commercial L. pomona bacterin only one month earlier. Agglutinin-absorption studies with LSU-91 and the Nebraska isolate indicated that the two organisms were homologous.

The serologic evidence of L. hardjo infections in cattle is widespread, but it is inconclusive. The actual reports of direct isolation are limited in the United States and the relationship of infection with clinical illness is still not understood. The recovery of Leptospira hardjo directly from infected host animals has proven difficult. This limits the documentation of L. hardjo infections by relying on serologic surveillance as the only indication of its prevalence in animal infections.

The objective of this thesis was to conduct a multifaceted investigation of the nutritional and cultural characteristics of Leptospira hardjo. Emphasis was placed on (1) the study of growth in albumin polysorbate (Tween) media; (2) defining the range of temperature over which the organism could be grown; (3) determining the viability of the organism when exposed to various temperatures; and (4) the effects of nutritional environment and temperature upon the immunogenicity in hamsters.

A laboratory adapted strain of L. hardjo was characterized as outlined above.

REVIEW OF LITERATURE

Historically, spirochetes were first observed in Japan in 1914 by Inada and were determined to be the causative agent of Weil's Disease (Inada et al. 1915). The organisms were cultured in a medium containing ascitic fluid and fresh sterile guinea pig kidney homogenate that had been used for the anaerobic cultivation of Borrelia.

Noguchi was the first to attempt to define the nutritional requirements of leptospires. He found that leptospires had an essential growth requirement for either human or animal serum, however, the growth capabilities varied from one species of leptospires to another (Noguchi 1918a,b,c). Heating the serum to 60° C for 30 minutes or boiling it reduced the growth promoting characteristics, however, filtration of the serum had little effect on the nutritional value. Noguchi found rabbit sera to be of good nutritional quality for the growth of Leptospira icterohaemorrhagiae and reported that supplementation of the rabbit serum media with carbohydrates did not improve either the rate, or total cell yield of the organism. He was able to determine that optimal growth occurred between 30° and 37° C with increased viability at 25° to 30° C under aerobic conditions.

Noguchi's methods were routinely used for several decades and were not improved upon until the 1940s. Serum containing media were used exclusively for the propagation

of leptospires and few attempts were made at defining the nutritional requirements of the spirochetes. Schuffner's Korthoff's and Chang's media were introduced, but they all relied upon a buffered salts system enriched with animal sera (Wolff 1954). An attempt was made to replace the essential serum factors in Schuffner's formulation by Greene (1945). When Schuffner's medium was deleted of heated rabbit serum the growth of L. canicola could not be sustained. Growth in the same medium enriched with serum could not be further stimulated by the addition of amino acids.

A medium formulated with ammonium chloride, asparagine, magnesium chloride, sodium chloride, glycerol, phenol red, and 10 percent rabbit serum proved superior to existing media. Stuart's medium (Stuart 1946) was simple to prepare and had better clarity and growth qualities than previously existed. He found that the quality of rabbit serum used varied from source to source and lot to lot. This made it mandatory to pool several serum samples and pretest each batch of complete media prepared. This medium proved reliable for the isolation of leptospires from animal and human sources, maintenance of laboratory stock cultures, and for use in the preparation of serologic antigens.

Stuart's contention related to the quality of rabbit serum was later reported by Johnson and Muschel (1966). They reported the L. biflexa or non-pathogenic serotypes wouldn't grow in the presence of normal serum of rabbit,

bovine, guinea pig, and human species mediated with the complement system. The eight pathogenic strains were susceptible to antibody plus complement but not to the normal serums.

Chang (1947) used a Warburg apparatus in order to define the nutritional requirements of L. icterohaemorrhagiae. He confirmed the facts that leptospires are obligate aerobes, show optimal growth at pH 7.2 and require serum for growth. He also concluded that simple sugars were not used and that the addition of glucose to the medium was not stimulatory. Unfortunately, his Warburg experiments were unable to demonstrate respiration by leptospires.

Marshall (1949) using manometric techniques, was able to show oxygen consumption by leptospires. A solution of inorganic salts and rabbit serum (8% by volume) stimulated respiration of L. icterohaemorrhagiae to nearly the same extent as whole Korthoff's media. However, he could not document that carbohydrate substrates were capable of stimulating oxygen uptake. He proposed two possibilities for the role of the serum fraction: (1) an important energy-producing substrate or co-factor existed or (2) that the increased respiratory activity was due to the removal of toxic substances by the protein binding of serum albumin. Marshall was also unable to demonstrate respiration with the use of carbohydrates, peptones, hemoglobin, or amino acids. This work was a major contribution in establishing

the role of serum and its nutritional qualities.

Greene et al. (1950) carried out extensive work in order to characterize the nutritional role of serum. Extensive dialysis did not reduce the activity of the serum, but when dialysed serum was treated with pepsin it was inactivated. Ammonium sulfate fractionation of dialysed rabbit serum also resulted in 80% less growth. They proposed that these studies indicated that the growth stimulating fraction of the serum was a protein. A semi-synthetic medium was prepared and was used to determine the vitamin and amino acid requirements for leptospires. Greene's formulation was comprised of dialysed rabbit serum, salts, vitamins, amino acids, and purine and pyrimidine bases. A requirement for thiamine was the only essential vitamin needed for the growth of Leptospira canicola.

Powelson and McCarter (1944) were able to demonstrate that the supplementation of serum albumin to a liquid medium would support the growth of the tubercle bacillus at a concentration of 1 cell/ml. Davis (1946) reported that 0.1% serum albumin (human or bovine) in a Tween medium grew the tubercle bacilli at low cell numbers confirming the findings of Powelson and McCarter. He proposed that the ability to bind fatty acids was a unique property of the serum albumin fraction. Further investigations revealed that the albumin interacts with the dializable components of the medium, and was also capable of neutralizing the toxic effects of the

free fatty acids supplied by the Tween.

Davis and Dubos (1947) found that each albumin molecule was capable of binding three to six molecules of oleic acid, thus preventing the inhibitory growth effects of the free fatty acids. Davis and Dubos proposed that the bacteriostatic effect of the commercial Tween preparation on some bacteria was due to the presence of unesterified fatty acids. A purified preparation of Tween 80 was not inhibitory to the growth of tubercle bacilli once the unesterified fatty acid was removed (Davis 1947). The addition of bovine serum albumin to media containing long chain fatty acids either reduced or completely eliminated the inhibition of bacterial growth. Albumin-enriched media supplemented with fatty acids often proved stimulatory to growth. Other proteins when added to the medium and fatty acids, failed to stimulate growth (Dubos 1947). The detoxification mechanism was explained as an affinity of the serum albumin for certain anions by Klotz (1949).

Taking the work of Davis and Dubos into consideration, Schneiderman et al. (1951) made important progress when they were able to replace whole serum with serum albumin in a chemically defined leptospiral medium. The authors used ammonium salt precipitation techniques to prepare albumin from rabbit, sheep, equine, and bovine serum for use in the defined medium. They were able to grow L. canicola in an albumin enriched medium containing 19 amino acids, vitamins,

purines, pyrimidines, sodium chloride, calcium, and potassium salts. When the albumin concentration was increased, the total growth of the leptospires was stimulated. The findings that the addition of 2.5 ml of rabbit albumin containing 6.9 mg of nitrogen proved as effective in promoting growth as 0.8 ml of dialysed rabbit serum altered further nutritional approaches.

Working on further refinements, Schneiderman et al. (1953) were unable to replace rabbit albumin with a mixture of amino acids. They also found that concentrations of rabbit albumin in excess of 10 mg % inhibited the growth of L. canicola. A simplified medium was formulated containing salts, thiamine, asparagine, and rabbit albumin. This medium allowed for the continuous subculture of L. canicola for two months. The other contribution of this work was the reconfirmation of the necessity for thiamine in order to sustain the growth of L. canicola.

An extensive re-examination of Marshall's work by Fulton and Spooner (1956) confirmed that the stimulatory effect of oxygen was dependent upon the presence of serum protein. They also demonstrated that fatty acids (acetic, proprionic, butyric, palmitic, stearic, and oleic acid) without a detoxifier were inhibitory to growth. Glucose and various amino acid mixtures were also shown to inhibit growth. They did not record any significant utilization of albumin as a protein source. This led them to attempt to

replace the albumin with a 5% suspension of activated charcoal. This substitution was unsuccessful.

Helprin and Hiatt (1957) used acetone precipitation to render human albumin fraction V lipid-free. This process reduced the growth stimulating activity of the fraction. However, when the extracted lipids were recombined with lipid-free fraction V, respiration was nearly identical to the unprocessed material. They concluded that the respiratory stimulating factor was lipid in nature but lipids alone proved inhibitory. Tween 80 had been documented as a source of oleic acid used for the growth of Mycobacterium tuberculiesis (Dubos 1947). Helprin and Hiatt were capable of stimulating the growth of L. icterohaemorrhagiae with Tween 80 at a concentration of 100 ppm without an exogenous protein. When lipid-free fraction V was supplemented with sodium oleate the results mimicked the whole fraction. Oleic acid alone inhibited respiration. This furnished evidence for their conclusion that the serum supplies detoxified fatty acids which are stimulatory to the respiration of leptospires. When a detoxifier was present in the medium, the amount of respiratory stimulation increased as the length of carbon skeleton of the fatty acids increased. In the absence of a detoxifier some of the saturated and all of the unsaturated fatty acids tested proved inhibitory to cellular respiration. These investigators proposed that the reason Fulton and Spooner failed to recognize that fatty

acids were stimulatory, was probably due to the use of sub-optimal concentrations of palmitic, stearic, and oleic acids.

Johnson and Wilson (1960) attempted to replace the rabbit serum fraction in hopes of preparing a completely autoclavable leptospira media. Whole rabbit serum was precipitated into three separate fractions: albumin, globulin, and whole serum ultrafiltrate, and analyzed for their growth-supporting activity. The globulin and ultrafiltrate preparations were unable to support the growth of L. pomona when used separately or combined. The albumin alone could support growth; but less than the complete rabbit serum. The albumin was also found to support growth when combined with the globulin and ultrafiltrate. The three fractions did not support growth when assayed separately. When all three fractions were recombined, good growth was obtained.

They were able to replace the albumin fraction with a weakly basic resin (Amberlite IR-45) and soluble starch. A three-fold function was proposed for albumin, a source of amino acids, a source of lipid material, and as an adsorptive detoxifier. When a 4% PPLO bovine serum fraction (Difco) was shown capable of replacing rabbit albumin, it indicated that the albumin plays a nonspecific role in the support of leptospiral growth.

Utilization of amino acids was reported by Gerhardt and Ball (1959). During a forty-eight hour incubation period,

the amino acid content of Schuffner's media containing L. canicola decreased compared to an incubated sterile control tube of media. Four amino acids were identified as being used: glycine, methionine, serine, and phenylalanine. Their work indicated that the formation of free fatty acids was due to a slow degradation of proteins, proteoses, and peptones that resulted in an increase in the amino acid levels present in the media.

Johnson and Gary (1963) used the acetone extraction methods of Helprin and Hiatt (1957) to extract albumin from serum for incorporation in a leptospira medium. Their intent was to evaluate the growth properties of fatty acids of various carbon chain lengths. Palmitic, heptadecanoic, stearic, and oleic acids proved capable of supporting growth when combined with free fatty acid-extracted rabbit albumin. Growth of seven pathogenic serogroups was supported by palmitic acid, which was preferred to the less soluble stearic acid. Toxicity was observed with linoleic and linolenic acid, however, oleic acid was once again shown to stimulate good growth of leptospires. C^{14}--labeled palmitic acid was shown to be incorporated into the cellular material of L. pomona indicating a direct utilization of fatty acids during growth. A simplified medium containing ammonium chloride, inorganic salts, thiamine, palmitic acid, and rabbit albumin proved useful for leptospiral propagation.

A modification of Stuart's medium (Ellinghausen and

McCollough 1965) was the next step in albumin-supplemented media. The authors fractionated Oleic Albumic Complex (Difco) and formulated a medium of sodium oleate, glucose and extracted bovine albumin. This formulation replied upon NH_4Cl as an added nitrogen source, while the asparagine, which is often contaminated with trace metals was deleted. When sodium oleate was added to the extracted albumin; the supportive role of the NH_4Cl was documented, the replacement for vitamin B_{12} was revealed and cell growth was supported. The medium supported the growth of 14 leptospiral serotypes without diminishing either growth or antigenicity through multiple subcultures over two years.

The Ellinghausen-McCollough media was reformulated by the addition of sodium pyruvate and glycerol (Johnson and Harris 1967a) for the study of leptospiral growth at low temperatures. The new modification has been routinely employed in numerous studies involving leptospires. Lipase activity was investigated with this medium by Ellinghausen and Sandvik (1965). Previously, the albumin fraction had been shown to be free of carbohydrates (Cohn et al. 1947) making this medium acceptable for the evaluation of the carbohydrate requirements of leptospires. Using a bovine albumin-polysorbate (Tween) 80 medium, Ellinghausen (1968) demonstrated the stimulatory effect of glucose of Leptospira pomona and nine other serotypes. The presence of carbon-14 labeled glucose was found in cellular fractions and indicated

that the metabolism of glucose occurs. Ellinghausen showed that the indogenous vitamin content of albumin is negligible, so this media could be used to determine the vitamin requirements of leptospires. This medium in a semisolid form proved superior to Stuart's and Fletcher's media for the successful isolation of L. grippotyphosa and other fastidious serotypes (Hanson et al. 1964).

The quality and shelf life stability of bovine albumin polysorbate 80 (BA-P80) media has been reported to be satisfactory for more than 36 months when stored at room temperature (25° C)(Ellinghausen, personal communication). Twenty-one of twenty-two strains stored in BA-P80 semisolid were viable when stored at room temperature for two years. The virulence of L. pomona (Ohio) remained unchanged after twenty-four continuous subcultures in liquid BA-P80 media and twenty-two subcultures in the semisolid form.

Johnson et al. (1969) compared the fatty acid requirements of pathogenic leptospires to those of the L. biflexa saprophytic groups. Using a fatty acid poor albumin preparation, they reported that leptospires were unable to elongate fatty acid chains. However, short chain fatty acid may be used in the presence of long chain fatty acids. The saprophytic strains tested were capable of using either saturated or unsaturated fatty acids. The pathogenic organisms could only use fatty acids with skeletons of 15 or 16 carbon atoms. Unsaturated fatty acids could only be metabolized in the

presence of saturated fatty acids. Again, free fatty acids were found inhibitory to pathogenic strains.

In review, the major role of the albumin fraction is the binding and detoxification of long chain fatty acids (Davis and Dubos 1946). Bovine albumin possesses six major binding sites and several minor ones (Spector et al. 1969). The ability of bovine albumin to bind different fatty acids varies, however, its binding capacity is better than rabbit or human serum albumin.

Bovine albumin does contain bound fatty acids, citrate, lactate, pyruvate, and low levels of glucose, asparate, and alphaketo glutarate (Hanson and Ballard 1968). Until the impurities can be removed, some investigators have chosen to prepare synthetic growth media in an attempt to clarify the nutritional requirements of leptospires.

Stalheim and Wilson (1964b) used the synthetic formulation of Vogel and Hunter (1961) to reconfirm the fatty acid requirements previously described by Johnson and Gary (1963). Forty-three strains consisting of 16 serotypes were screened in this formulation and scored for growth or lysis. They incorporated Tween 80, 60, and 40 into the synthetic formulation, and although all were capable of supporting leptospires, a mixture of Tween 60 and 80 proved optimal. Both vitamin B_1 and B_{12} were shown necessary for maximum growth of L. canicola. Their successful attempt at growing L. canicola in a medium containing fatty acids as the only carbon source

supported the hypothesis suggested by Johnson and Gary (1963).

An autoclavable serum-free medium of inorganic salts, vitamins B_1 and B_{12}, Tween 80, tryptosephosphate and agar was developed which could support growth of a saprophytic leptospire isolate designated B-16 (Baseman et al. 1966). Equivalent cell yields were obtained when palmitic and oleic acid equivalent by weight to the fatty acid concentration in Tween 80 replaced the Tween in this synthetic media. In this medium, ammonium ion served as the sole nitrogen source. Vitamin B_{12} was required and the addition of thiamine resulted in a 60% increase in growth.

A second serum-free chemically-defined medium capable of supporting the continuous subculture of 52 and 61 strains was reported (Shenberg 1967). This basal medium contained inorganic salts, a fatty acid, and vitamins B_1 and B_{12}. Good cell yields similar to serum containing media were observed. Ammonium chloride proved adequate as a nitrogen source, but asparagine was better for the strains assayed. Shenberg concluded that asparagine was used as both a carbon and nitrogen source. The medium was practical for defining nutritional requirements of L. canicola, L. pomona, and L. grippotyphosa. Vitamin B_{12}, iron, and fatty acids were essential nutrients for growth. The defined medium was successfully used for the isolation of leptospires from infected hamster tissues diluted to small numbers. The synthetic media has obvious advantages for the growth of leptospires for the preparation of diagnostic reagents and

vaccines, however, selective antigenic changes have been reported (Stalheim and Wilson 1964a).

The latest synthetic or reduced serum protein medium was introduced by Bey and Johnson (1978). Using charcoal extraction techniques developed by Chen for extracting fatty acids from albumin, Bey and Johnson attempted to replace the albumin fraction by detoxifying the Tween before addition to the basal media. Although attempts at detoxifying the Tween by passage through anion exchange columns with various resins proved unsuccessful, the actual addition of the resins to the medium was satisfactory for the propagation of leptospires. The antigenicity of five serovars was evaluated and showed no appreciable difference through nine continuous subcultures. The isolation capabilities of the protein free media was evaluated with four hamster lethal leptospiral strains. Samples of blood from infected hamsters were inoculated into protein free and BA-P80 media and observed for viable organisms. Within four to six days, growth was detected in both media, however, with dilute inocula (10 cells/ml or less) growth occurred several days earlier in BA-P80 media.

Many enzymes have been described for leptospires. Catalase (Faine 1960), oxidase (Goldberg and Armstrong 1959), and transaminase (Markovetz and Larsen 1959) are present in leptospires. An analysis of intracellular and extracellular enzymes was made of 37 serotypes (Green et al.

1967). They found various patterns of mobility and degree of extracellular esterase using starch gel electrophoresis techniques. The presence of catalase, phosphatase, and naphthylamidase was not demonstrated in any extracellular preparations. Intracellular examinations of several strains found previously unreported enzymes including esterases, phosphatases, lactic, malic, glutamic, succinic, alpha-glycerolphosphate, 6-phosphagluconic dehydrogenases, and naphtylamidase. These enzymes suggest the existence of tricarboxylic acid, glycolytic and pentose related pathways. This was confirmed (Baseman and Cox 1969) and acylcoenzyme A dehydrogenase required for beta-oxidation of fatty acids was also described.

Chang et al. (1948) reported the viability of leptospires in water at neutral pH to be a function of the temperature of the water. They reported that L. icterohaemorrhagiae survived 8 to 9 days at 5° to 6° C and only to 4 days at 31° to 32° C. Generation times were slower at low temperatures, however the organisms remained viable for a longer time. High temperatures shortened the generation time but drastically reduced the total life span. The thermal death point of L. icterohaemorrhagiae was determined to be 45° C for 20 to 30 minutes, 5 to 10 minutes at 50° C, and only 10 seconds or less at temperatures higher than 60° C.

The ability to grow at selected temperatures was used to differentiate saprophyte strains from pathogens (Johnson

and Harris 1967a). Pathogenic leptospires were found capable of growth between 13° to 15° C, while the saprophytes could grow at temperatures approximately 8° C lower. *In vitro* cultivation normally occurs at 28° to 31° C for optimal growth, however, temperatures as low as 20° to 23° C (room temperature) may be used depending on the serotype and strain.

Ellinghausen (1971) tested 28 strains for the ability to grow in continuous subculture at 37° C. He found the ability to grow at this elevated temperature varied *in vitro* and could not be correlated with virulence in hamsters. All *L. grippotyphosa* strains were temperature sensitive at 37° C, while *L. pomona* varied among the strains. Two *L. icterohaemorrhagiae* and one *L. biflexa* strain grew continuously at 37° C.

Leptospiral serotypes vary widely with respect to virulence. Virulent and avirulent strains of the same serotype show no serological difference (Faine 1957a; Morton 1961). The significance is the ability of the virulent organism to survive and replicate in the infected host (Faine 1957a). *In vivo* growth of *L. icterohaemorrhagiae* in young guinea pigs suggested that virulence is the result of differential survival (Faine 1957b). He reported a constant relationship between survival time and the dose.

Changes in virulence may be the result of the environment of a previous host. Virulence of blood and urine isolates from swine did not undergo alterations, however did

differ depending whether the organisms were isolated from blood or urine in cattle. The previous host species, and the duration of time spent in the host influenced the virulence of an isolate passed in artificial media. Blood isolates increased in virulence with passage in artificial medium (Chrisp and Ringen, 1962).

Nutritional factors may alter the virulence of an organism (Stalheim 1966). He reported a decrease in virulence after passage in artificial media. Repeated subculture in Shenberg's medium also decreased the virulence (Kida 1969). Johnson and Harris (1967b) reported a correlation between the reversion to avirulence and the loss of resistence to the antibody-complement complex. They showed that virulent leptospires were capable of replication in the presence of antibody and complement, but the rate of multiplication and the amount of antibody produced was critical. The appearance of enough antibody early in the course of infection removes the organisms before renal infection is established. When 10^8 organisms of an avirulent culture were injected in hamsters no infection occurred. However, the injection of 10^6 organisms produced renal infection. The authors postulated that the large number of organisms stimulated the production of sufficient antibody to prevent renal infection.

Leptospires are known to enter the host animal through skin abrasions or by penetration of mucous membranes of the

eye, genital tract, or mouth (Hanson 1976). The replication of the organisms occur in the liver and other organs followed by leptospiremia. Localization of the infection appears to reside in the kidney and brain tissue for extended periods of time.

Clinical signs of infection include anemia, jaundice, hemorrhages, hemoglobinuria, and agalactia in cattle (Reinhard and Hadlow 1954). The major effects of leptospires are focused in the kidneys causing nephritis and chronic lesions (Hadlow and Stoenner 1955). Shedding of viable organisms may persist for several months in cattle (Sullivan 1970). Leptospiuria will vary with the host animal and with the infecting serovar. Abortions and infertility problems are the major complication in cattle and swine after an acute illness of leptospirosis.

The pathogenesis of L. hardjo differs from the other leptospires. The initial isolation by Roth and Galton (1960) was made from clinically healthy cattle. Subclinical infections are often reported in dairy herds after a drop in milk production (Sullivan 1970). A herd infection incidence of 14.8% due to L. hardjo is reported in Australia based on serological evidence.

Abortion of fetuses during the last third of gestation is associated with clinical leptospirosis. Hanson and Brodie (1968) reported two pregnancies in cattle terminated after experimental infection with L. hardjo. The mechanism

is probably due to the invasion of the fetus by leptospires. Others have proposed that early cases of leptospirosis prevent proper implantation of the embryo in the uterus.

The inability to recover the fastidious leptospires like L. hardjo places a strong emphasis on serologic tests to determine the prevalence of this serotype. The leptospires are all serologically related and cross-react in serologic tests. Rothstein and High (1957) describe two distinct antigens; a heat stable genus specific antigen and a heat-labile serotype specific antigen. The serotype-specific agglutinating reactions appear to be a characteristic of the cell wall (Faine 1965). The genus specific hemagglutinating and complement-fixation reactions were found in the surface layer. These findings were later substantiated by Anderson and Johnson (1968). More recent work (Palit et al. 1974) has shown the type-specific antigen to be inherent to the outer envelope.

The live antigen microscopic agglutination test is the most widely used diagnostic procedure for the diagnosis of leptospirosis (Galton et al. 1962). Other tests were developed for the identification of unknown serotypes, but are not as popular for various reasons. Variations of the test do exist. Standardization of the antigen density, time, and temperature of incubation has not been made. The use of formalinized antigens may be used, however, the titers will be two-fold to four-fold lower (Alexander 1976).

A microtiter test modified to use standard microtiter equipment proved feasible. Less antigen volume is necessary and the multiple dilutions may be made in the disposable plates (Galton et al. 1965). Further refinements (Cole et al. 1973) make use of a long-working distance objective so that the test is interpreted directly in the plastic plate.

Nutritional factors have been reported to alter the agglutination pattern of leptospires (Stalheim 1965). He found organisms grown in Stuart's medium reacted differently to those grown in a chemically defined medium. Seven subcultures in Stuart's medium were required to restore the antigenicity to cells grown in the defined culture medium. Ellis (1970) reported a difference in the agglutination of leptospires grown in polysorbate substituted bovine albumin media. However, Roberts (1972) saw no appreciable difference in the agglutination of L. icterohaemorrhagiae grown in fourteen different substituted and deleted media. He offered evidence that the micronutrients bound by the albumin moiety along with vitamins B_1 and B_{12} were sufficient to synthesize the antigenic determinants of the organism.

A cattle bacterin using formalinized leptospires was reported by Olitzki et al. (1949). Their work showed that formalin treated cultures provided a moderate but not solid immunity. A bacterin described by York and Baker (1953) was produced and tested for the immunization of cattle against Leptospira pomona. Their bacterin differed from all

others in that it was propagated in embryonated eggs. Brown et al. (1954) modified this bacterin by culturing L. pomona strain T 262 in Stuart's medium. The growth of strain T 262 was equal in both sources, however, it was more immunogenic when grown in culture media. The addition of small quantities of thiamine increased the growth four- to six-fold while improving the antigenicity.

The outer sheath of leptospira has been shown to have immunologic properties that protected as well as whole cell preparations (Auran et al. 1972). An immunizing dose of 1.0 µg dry weight protected hamsters against death and prevented renal infection. Foxes vaccinated with two 46 µg doses of an outer envelope bacterin were protected against clinical and renal infections when challenged with 2.6×10^9 cells (Glosser et al. 1974). Protection against kidney infection is important in the control of leptospirosis. Once renal infection is established, the carrier state makes further transmission of the infection to healthy animals possible. Bey et al. (1974) compared whole cell and outer envelope bacterins by two parameters; protection against death and protection against kidney infection. Their results showed that higher levels of vaccine of either type were necessary to protect against kidney infection than were needed to protect against death. The authors also concluded that virulent pathogens produced better bacterins than avirulent pathogens in both types of vaccines. The

immunogenic potential of outer envelope bacterins from virulent pathogens was equal to, or better than, the homologous whole cell preparations. Painter and Ellinghausen (1976) found that heat inactivated whole cell bacterins of an avirulent L. canicola strain protected as well as a virulent strain preparation. Vaccination of hamsters with 10 to 25 µg protected against infection and death while a 40 µg dose prevented leptospiruria. No difference was seen between bacterins prepared with organisms exposed to temperatures of 50° to 98° C. Cells inactivated at 121° C gave protection against lethal infections, but not against leptospiruria. They found no local or systemic reactions to heat-killed bacterins in agreement with previous reports (Alston and Broom 1958). Zeigler et al. (1976) demonstrated heat-killed whole cell and outer envelope bacterins of virulent and avirulent leptospires to be good immunogens. Their avirulent preparations did not protect against renal infections at low dosages. At the 1 µg/ml concentration, the heat-killed whole cell bacterins were more effective than outer-envelope vaccines.

MATERIALS AND METHODS

Cultures. The following serotypes Leptospira hardjo (Hardjoprajitno), L. canicola (Hond Utrecht IV), L. icterohaemorrhagiae (M-20), L. pomona (DM_2H), and L. grippotyphosa (Moskva V) were supplied through the courtesy

of Dr. H. C. Ellinghausen, Jr., of the National Animal Disease Center, Ames, Iowa. The stored semisolid cultures were maintained in 10 ml of bovine albumin polysorbate 80 semisolid medium in 20 x 125 mm screw-capped tubes at room temperature (20-26° C). A liquid culture inocula was prepared by subculturing 2.0 ml from the visible growth region (Dinger's Zone) in semisolid media to 9.0 ml of a second tube of BA-P80 semisolid medium. Fourteen days later 1.0 ml was transferred to 9.0 ml of liquid BA-P80 medium. When the macroscopic turbidity was relatively dense (5 to 7 days), 1.0 ml was subcultured to a new tube of liquid medium. In order to reduce the amount of residual agar, a third 1.0 ml subculture in 9.0 ml of liquid medium was made. A battery of the five serotypes was maintained in liquid medium and was subcultured every fourteen days for the length of this study.

Measurement of growth in liquid media. Growth of leptospires in liquid media was measured by the use of a Coleman 9 nephelocolorimeter (Coleman Instruments, Maywood, Illinois), a NADC secondary turbidity standard (Ellinghausen 1976), and a NACD dry well. Growth in semisolid media was evaluated by the appearance of a macroscopic zone of increased turbidity, a Dinger's Zone, when tubes were examined by an intense white light.

Selection of the secondary standard. Four secondary standards of different nephelos ratings 0, 31.5, 50.5, and 115 were compared. One ml from a four-day-old liquid culture adjusted to a nephelometer reading of 25 was transferred to a fresh tube of liquid BA-P80 medium. The nephelometer was adjusted to the various ratings and growth was recorded over seven days.

Selection of a dry well. Three aluminum dry well prototypes (NADC, Ames, Iowa) were produced according to a design of Roessler and Brewer (1967). One was coated inside and out with an aluminum ink (Doall Company, Des Plaintes, Illinois), only the interior was darkened in the second and the third was not treated with the ink but anodized to give the well a black matte non-reflective surface. The three were evaluated against the water well while recording the growth of leptospires over a seven-day period.

Growth measurement using nephelometry. A Coleman 9 nephelocolorimeter was employed to measure the increase of turbidity of all leptospira cultures. A NADC secondary standard rated at 50.5 nephelos was used to adjust the machine by the direct method of measurement as described by the manufacturer.

Scale expansion of secondary standards. The titanium dioxide secondary standards were used to adjust the nephelometer to any one of four values. The rated value

was used in most circumstances, one-half of the rated value was used to measure old or concentrated cultures. The same standard could adjust the instrument to one and one-half or twice the rated value for measuring the turbidity of young or dilute suspensions.

A comparison of three standards rated at 31.0, 50.5, and 115 using scale expansion was made.

Nephelometry vs. percent transmission. One ml of a four-day-old culture of L. hardjo was adjusted to 25 nephelos and inoculated into fresh (room temperature stored at 25° C) BA-P80 media. The growth was measured daily by a nephelometer adjusted to 50.5 nephelos by the direct method. These values were compared to percent transmittance of the Coleman 9 colorimeter using filters rated at 390, 525, and 635 nm over a seven-day period at 29° C.

Media. Bovine albumin polysorbate 80 medium (BA-P80) was employed for growth, isolation, and maintenance of the leptospires used in this study (Ellinghausen and McCollough 1965). It is a serum-free medium using bovine albumin fraction V powder (lot 231, Miles-Pentex Laboratories, Kankakee, Illinois) plus polysorbate 80 (Tween 80, Atlas Powder Company, Wilmington, Delaware). The formula for the media consists of a phosphate buffered salt solution of ammonium, magnesium, and sodium chlorides, copper, iron, and zinc sulfates, vitamin B_1 and B_{12}, polysorbate 80

(sorbitan monooleate), and a 1% concentration of bovine serum albumin.

Preparation. To 700 ml of glass distilled water were added 40 ml of 25x phosphate buffer, 50 ml of 20x salts, 1 ml of copper solution, 10 ml of zinc solution, and 20 ml of iron solution. This mixture was stirred for at least five minutes. Two hundred mg of L-cystine was added and stirred for three minutes. No attempt was made to completely dissolve the L-cystine. The solution was filtered through a triple thickness of Whatman #1 filter paper. Twenty ml of vitamin B_{12} working solution and 0.1 ml of Vitamin B_1 working solution was added. To this filtered volume was added 120 ml of Tween 80 (1% v/v) and adjustment to 1000 ml was made with distilled water. This same formula common for liquid, semisolid, and solid plating media is given in Table 1.

Preparation of bovine serum albumin. Five grams of Bovine albumin fraction V powder were dissolved in 100 ml of single strength sterile phosphate buffer. The solution was filter-sterilized by drawing it through a 0.22 micron membrane filter (Nalge Sybron Corporation, Rochester, New York) at 3 psi. One ml of the filtrate was inoculated in trypticase soy broth at 37° C to test the sterility of the albumin. The remaining albumin was also incubated at 37° C for 24 hours and then stored at room temperature. It

Table 1

Composition of One Liter of Basal Solution

Stock Solution	Volume Added	Concentration of Stock Components
25x phosphate buffer	40 ml	16.6 g/l Na_2HPO_4 2.17 g/l KH_2PO_4
20x salt solution	50 ml	38.5 g/l NaCl 5.35 g/l NH_4Cl 3.81 g/l $MgCl_2 \cdot 6H_2O$
Copper solution	1.0 ml	0.30 g/l $CuSO_4 \cdot 5H_2O$
Iron solution	20.0 ml	2.5 g/l $FeSO_4 \cdot 7H_2O$
Zinc solution	10.0 ml	0.40 g/l $ZnSO_4 \cdot 7H_2O$
L-cystine	200 mg	
Vitamin B_{12} concentrate		1.0 mg/100 ml H_2O
Vitamin B_{12} working solution	20 ml	10 ml concentrate + 90 ml H_2O
Vitamin B_1 solution	0.1 ml	200 mg/100 ml
Tween 80 solution	120 ml	10 ml/liter
Bovine albumin (5% solution)		100 ml/liter of complete basal

is judicious to assess the bacterial flora of BSA in order to aid in monitoring of the effects of filtration procedure.

Liquid media. The basal media was made according to the formula previously described. Eight ml was dispensed into 19 x 150 mm tubes with #20 Morton Closures (Bellco Glass Company, Vineland, New Jersey). The incomplete basal media (exclusive of albumin) was sterilized at 121° C at 15 pounds pressure for 15 minutes. Two ml of a filter-sterilized solution of 5% BSA in single strength phosphate buffer was added to each tube when cooled to 56° C. The tubes were sealed with Parafilm (American Can Company, Neenah, Wisconsin) and stored at room temperature.

Semisolid media. The semisolid media was prepared by adding 2.5 g of agar to one liter of BA-P80 basal. The medium was heated to 100° C to completely dissolve the agar. Eight ml was dispensed into 20 x 125 mm screw capped tubes (Bellco Glass Company, Vineland, New Jersey) and sterilized at 121° C at 15 psi for 15 minutes. The tubes were equilibrated to 56° C in a water bath and 2.0 ml aliquots of BSA were added. The final concentration of agar is 0.2%. The tubes were sealed with black electrical tape and stored at room temperature.

Solid plating medium. A plating medium was prepared by the addition of 12.5 g of agar to one liter of BA-P80 basal. The agar-basal solution was heated to 100° C and

mixed to dissolve the agar. The medium was sterilized as described and cooled to 56° C. Aseptically 250 ml of 5% BSA was added to the agar-basal solution. The final agar concentration is 1% in the completed solid medium. Thirty ml aliquots of medium were poured into sterile 10 cm glass petri plates. The plates were stored at 5° C until used.

Deleted media. Four deletion media were prepared by eliminating essential ingredients in both liquid and semi-solid media. The four media deleted one of the following: Tween 80, ammonium chloride, vitamin B_1, or vitamin B_{12}.

Additional deletions were made in liquid media: L-cystine, trace metals (Copper, Iron, Zinc), trace metals and L-cystine, and a triple deletion of polysorbate 80, ammonium chloride, and L-cystine.

Simplified semisolid medias also included:

0.2% agar in 1x phosphate buffer

0.2% agar in 1x phosphate buffer and 1% BSA

0.2% agar in 1x phosphate buffer, 1% BSA, and vitamin B_{12}

0.2% agar in 1x phosphate buffer, 1% BSA, and vitamin B_1

0.2% agar in 1x phosphate buffer, 1% BSA, and vitamin B_1 and B_{12}.

These media lacked NH_4Cl, $MgCl_2$, NaCl, trace metals, L-cystine, and Tween 80.

Substitution media. Polysorbate 60 (Sorbitan monostearate), polysorbate 40 (sorbitan monopalmitate), and

polysorbate 20 (sorbitan monolaurate) were each substituted for polysorbate 80 in liquid and semisolid media.

Standardization of media. All media were tested for the ability to support the growth of diluted cell numbers. Nephelometer readings were used to follow progressive growth of L. hardjo. All cultures were calibrated to 25 nephelos which approximates 3.4×10^8 cells/ml (Ellinghausen, personal communication).

The inocula were serially diluted from 10^{-1} to 10^{-10} and observed for turbidity.

Standardization of inocula. A Coleman 9 nephelocolorimeter and a secondary turbidity standard were used to calibrate cultures in liquid media. The cultures were adjusted to 25 nephelos units by diluting the organisms in 1x phosphate buffer using Table 2. The cultures were checked with the nephelometer to insure that the dilution procedure was correct.

Cell counts. Total cell number estimates were done by direct counts under dry darkfield microscope (Ernest Leitz, Wetzlar, West Germany) at a magnification of 250X. A Petroff-Hausser counting chamber (Hausser Scientific, Blue Bell, Pennsylvania) was used according to established procedures. A 25 NR standardized culture diluted 1:10 was found best for direct counts. Duplicate or triplicate

Table 2

Diluent Volumes for Adjustment of Leptospiral Antigen Suspensions to a Nephelometer Reading (N.R.) of 25

N.R.	N.R./25	Culture Vol. MLS. 10 ML/F	Diluent Vol. MLS. 1% BSA
100	4F*	2.5	7.5
90	3.6	2.8	7.2
80	3.2	3.1	6.9
70	2.8	3.6	6.4
60	2.4	4.2	5.8
50	2.0	5.0	5.0
40	1.6	6.2	3.8
30	1.2	8.3	1.7

*F = N.R. of actual culture divided by 25 N.R. (desired suspensions turbidity; Coleman 7 or 9).

counts were made and the average number of organisms was determined.

Growth in liquid media. A culture of L. hardjo in BA-P80 medium was adjusted to 25 nephelos with a Coleman 9 nephelocolorimeter. One ml of the standardized culture was inoculated in 10 ml of each of the following liquid media:

Polysorbate 80 medium

Polysorbate 60 medium

Polysorbate 40 medium

Polysorbate 20 medium

Polysorbate 80 deleted medium

Ammonium chloride deleted medium

Vitamin B_1 deleted medium

Vitamin B_{12} deleted medium

L-cystine deleted medium

Trace metals deleted medium

Trace metals and L-cystine deleted medium

Polysorbate 80, ammonium chloride, and L-cystine deleted medium.

Ellinghausen-McCollough - Johnson-Haris Modification (EMJH Difco Laboratories, Detroit, Michigan), Stuart's medium, oleic-albumin complex medium, and asbestos filtered BA-P80 medium were also evaluated. The cultures were incubated at 29° C for fourteen days and turbidity was measured daily by nephelometry.

The sixteen liquid media were tested for their ability

to support the growth of a diluted cell inocula. The 25 NR culture was serially diluted 10^{-1} to 10^{-10} in each of the media. The tubes were incubated statically at 29° C for twenty-one days and observed daily for the appearance of macroscopic turbidity.

The ability of the media to support continuous subculture was also evaluated. The first tube (34×10^{6} cells/ml) and the last tube in the serial dilution that grew to a maximum nephelometer reading were used. One ml of each tube was readjusted to 25 NR with sterile 0.005M phosphate buffer diluent and inoculated into a fresh tube of the same culture media. One ml of unadjusted culture was continuously subcultured to determine if readjustment with phosphate buffer affected the ability to subculture indefinitely. Both sets of newly inoculated tubes were incubated at 29° C for ten days and the turbidity was measured. The various media were subcultured in this manner sufficiently to establish the ability of the medium to support continuous growth.

Each subculture was tested for viability by transferring 1.0 ml of each culture to a tube of BA-P80 semisolid media. The tubes were incubated at 29° C for twenty-eight days. The appearance of a Dinger's zone indicated the presence of viable leptospires. Darkfield examinations were made when no growth was visible.

Growth in semisolid media. A culture of L. hardjo in BA-P80 liquid medium was standardized in the nephelometer. The culture was serially diluted from 10^{-1} to 10^{-10} in sterile 0.005M phosphate buffer. Each of the following semisolid media was inoculated with 1.0 ml of each dilution:

Polysorbate 80 medium

Polysorbate 60 medium

Polysorbate 40 medium

Polysorbate 20 medium

Polysorbate 80 deleted medium

Ammonium chloride deleted medium

Vitamin B_1 deleted medium

Vitamin B_{12} deleted medium

These cultures were incubated at 29° C for twenty-one days and observed daily for the appearance of a Dinger's zone.

Several simplified semisolid media were evaluated in the same way. These media were:

Agar + phosphate buffer

Agar + phosphate buffer + 1% BSA

Agar + phosphate buffer + 1% BSA + vitamin B_1

Agar + phosphate buffer + 1% BSA + vitamin B_{12}

Agar + phosphate buffer + 1% BSA + vitamin B_1 and B_{12}

Agarose 0.2% + phosphate buffer + 1% BSA + vitamin B_1 and B_{12}

Agarose 0.1% + phosphate buffer + 1% BSA + vitamin B_1 and B_{12}

The simplified semisolid media were incubated for 21 days at 29° C. At the end of three weeks, each tube was examined under darkfield at 250X to verify the viability of leptospires; since under such minimal growth conditions the development of visible growth was limited.

Evaluation of agar products. Nineteen samples of agar from various sources were used to prepare BA-P80 semisolid media as described. A culture of L. hardjo was adjusted to 25 nephelos, and serially diluted 10^{-1} to 10^{-10} in sterile 0.005M phosphate buffer. Each of the agars were inoculated with 1.0 ml of the culture to test their ability to support the growth of dilute cell inocula. The cultures were incubated at 29° C for twenty-eight days. They were checked every seven days for the appearance of a Dinger's zone. All negative tubes were examined microscopically at 250X for the presence of leptospires.

Evaluation of albumin products. Eighteen varieties of albumin were filter-sterilized as 5% solutions in sterile 0.005M phosphate buffer. They were added aseptically to liquid BA-P80 media and evaluated for their ability to grow leptospires. L. hardjo was standardized to 25 NR and 1.0 ml was transferred to media supplemented with the following albumins:

Fatty acid poor lot #7

Fatty acid free lot #163

Lot 163

Crystalline lot #23

Lot #29 at pH 7.2

Lot #29 at pH 7.4

Lot #29 at pH 7.6

Lot #29 at pH 7.8

Lot #29

Rabbit albumin

Porcine albumin

Equine albumin

Reheis Leptel 7

Gibco albumin

Wilson lot #1

Wilson lot #2

Wilson lot #3

Phillips-Roxanne commercial media

Miles Pentex bovine albumin lot #231

The media were also tested for their ability to grow a small cell number inocula. A 25 nephelos culture was diluted 10^{-1} to 10^{-10} in sterile 0.005M phosphate buffer. One ml was inoculated into each tube. The static cultures were incubated at 29° C for 21 days and the growth was measured by nephelometry every seven days.

The growth of L. hardjo in gamma irradiated BSA. Bovine albumin fraction V powder lot #261 was gamma irradiated at two dosages, 1.25 and 2.50 megarads by Dr. Dave

Lairadett (Tasmin Vaccine Co., Upper Hut, New Zealand). The albumins were filter-sterilized as 5% solutions in 0.005M sterile phosphate buffer. They were added aseptically to liquid BA-P80 media and evaluated for growth promoting ability through ten continuous subcultures. The cultures were readjusted to 25 NR every seven days and subcultured into fresh media. Nephelometric techniques were used to monitor progressive growth.

The effect of various concentrations of BSA on growth. Albumin lot 231 was filter-sterilized in various concentrations in 0.005M phosphate buffer as follows: 0.25%, 0.50%, 0.75%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 10%, 15%, 20%, and 25%. A 2X BA-P80 basal media was prepared and 4.0 ml was dispensed into 19 x 150 mm tubes. An additional 4.0 ml of distilled water was added and the 8.0 ml volume was sterilized at 121° C at 15 pounds for 15 minutes. Two ml aliquots of the appropriate concentration of BSA was added to the basal. Two ml of distilled water was added in place of BSA for a zero percent concentration blank.

One ml of a 25 NR standardized culture was added to a tube of each concentration and incubated at 29° C. One ml subcultures to fresh media were made every seven days through five passages.

The effect of temperature on growth. Experiments were designed to determine the lowest and highest temperature

which would support the growth of L. hardjo. A culture was adjusted to 25 NR with a Coleman 9 nephelocolorimeter and 1.0 ml inoculated into tubes of BA-P80 liquid medium. Three tubes were incubated at each of the following temperatures: 7°, 13°, 14°, 16°, 23°, 29°, 31.5°, 35°, 37°, 38°, 39°, and 40° C. Growth was measured daily for two weeks to determine an average growth curve for each temperature. The ability of the organisms to grow in continuous subculture was also evaluated. Each week 1.0 ml from all tubes was inoculated into fresh BA-P80 liquid media and returned to the respective temperatures. Growth was measured daily through three subcultures.

Selected temperatures (7°, 13°, 23°, 29°, 37° C) were tested for the ability to grow a dilute cell inocula. A standardized culture was diluted 10^{-1} to 10^{-10} in BA-P80 liquid media and incubated for 21 days at these temperatures. The growth of the cultures was measured by nephelometry. At the end of 21 days, one ml of each tube was placed in BA-P80 semisolid medium to determine the viability of the leptospires. The semisolid cultures were incubated at 29° C for three weeks. The presence of a Dinger's zone was accepted as a viable culture of leptospires.

Initiation of growth at 37° C. In order to study possible correlation between growth at 37° C and changes in antigenicity, immunogenicity, and pathogenicity it was necessary to grow sufficient numbers of organisms at this

elevated temperature. A four-day-old culture incubated at 37° C was adjusted to 25 nephelos with a nephelometer. A 1:10 dilution was made in 1% BSA and a cell count estimate made after triplicate counts with a Petroff-Hausser cell counting chamber. Ten-fold dilutions were made in BA-P80 liquid medium from 3.4×10^6 cells/tube. The cultures were incubated at 37° C for 21 days and turbidity was measured daily. The viability of the organisms was determined in the manner described.

Growth of L. hardjo on solid agar plates at 29° and 37° C. Two cultures, one incubated at 29° C and one at 37° C were standardized at 25 NR, and diluted from 10^{-1} to 10^{-10} in sterile 0.005M phosphate buffer. Two-tenths ml from each dilution of the two cultures was placed on a plate of BA-P80 solid plating medium. The inoculated plates were swirled for one minute to disperse the inoculum. Each plate was sealed with masking tape and incubated at the original temperature in an inverted position. The plates were observed every seven days for the appearance of opaque subsurface colonies.

The effect of temperature on antigenicity. Leptospira hardjo cultures grown at 29° and 37° C in liquid medium were tested by the microscopic agglutination test for a comparison of antigenicity. Each antigen was reacted with the following antiserum:

L. hardjo (Hardjoprajitno)

L. hardjo (Downey)

L. hardjo BV 6 (Canadian isolate)

L. wolffi (3705)

L. sejroe (M-84)

L. swajizak (CDC strain)

and antisera from members of the hebdomadis serogroup. Cross agglutination tests were determined by reading the antigens against antisera of the following serotypes:

L. copenhageni (M-20)

L. naam

L. poi

L. coxi

L. canicola (Hond Utrecht)

L. ballum

L. pyrogenes

L. autumnalis

L. pomona (DM_2H)

L. grippotyphosa (Moskva V)

The microscopic agglutination test was performed according to the following procedure:

a. A four-day-old leptospiral culture was adjusted to a reading of 25 nephelos with a nephelometer.

b. A 1:5 dilution was made by transferring 0.2 ml of serum to a 75 mm tube containing 0.8 ml of 0.85% phosphate-buffered saline.

c. Serial dilutions were made transferring 0.1 ml from the 1:5 dilution to successive tubes containing 0.9 ml of saline. This resulted in dilutions of 1:5, 1:50, 1:500, 1:5,000, and 1:50,000. A 0.2 ml volume was removed from the tubes and was placed in wells of a serology plate.

d. The standardized antigen was dispensed in 0.2 ml volumes to each well giving final dilutions of 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000.

e. A negative control of 0.2 ml of known non-infected human serum and 0.2 ml of antigen was reacted in each series to check for spontaneous agglutination. An antigen control tube of 0.2 ml of saline and 0.2 ml of antigen was used to insure the quality of the antigen and determine the amount of breed clumps. The wells were incubated at room temperature for one hour.

f. Each dilution was read by darkfield examination at 100X. The agglutination was rated at 1+ (25% agglutination) to 4+ (100%) when compared to the antigen control.

Heat-killed bacterin preparation. Ten ml of a 25 NR L. hardjo culture was added to 100 ml of BA-P80 liquid media in each of ten 250 ml nepheloculture flasks (Bellco Glass Incorporated, Vineland, New Jersey). Five flasks were incubated at 29° C and five at 37° C. The cultures were

measured daily with a nephelometer for seven days.

Preliminary studies indicated that one hour exposure at 65° C in a water bath would render the organisms nonviable. After the flasks had been heat treated, one ml from each flask was transferred to tubes of BA-P80 semisolid media to check for viability.

The cultures were centrifuged at 35,000 rpm in a Sorvall centrifuge (DuPont Company, Wilmington, Delaware) 45 minutes. The pellet of both bacterins was washed three times in 0.005M phosphate buffer. The final two pellets were resuspended in 5 ml of buffer.

The two bacterin suspensions were characterized by a Beckman DU Spectrometer (Beckman, Fullerton, California) at 400, 500, and 600 nm and with a Bausch and Lomb Spectronic 20 (Bausch and Lomb, Rochester, New York) at 400, 500, and 600 nm, a Coleman 9 colorimeter with filters of 390, 525, and 655 nm and a Coleman 9 nephelometer. Scale expansion was used to determine the turbidity of the cultures.

Dry weights were determined for both bacterins and each was diluted in 1% BSA to a concentration of 10 mg/ml. The sterility was checked by inoculating 1.0 ml of each in 10 ml of trypticase soy broth at 37° for seven days. The bacterins were dispensed into separate sterile glass containers, sealed with aluminum-capped rubber stoppers and frozen at -70° C until used.

The effect of culture in different Tweens on antigenicity. L. hardjo cultures grown in bovine albumin media containing one of the four Tweens 80, 60, 40, and 20 were tested by microscopic agglutination for possible differences in their antigenicity. The microscopic agglutination test was performed as previously described.

Agglutinin development from 29° C grown heat inactivated bacterins. Forty-eight male weanling Syrian hamsters were purchased from Engles' Laboratory in Farmersburg, Indiana. Twenty-four animals were inoculated intraperitoneally with 1.0 ml (10 mg/ml) of a heat inactivated bacterin. The hamsters were sacrificed every two days post-inoculation and blood was collected by cardiac puncture. The blood was allowed to clot 24 hours at 7° C, then processed for serum and frozen.

Agglutinins were assayed by means of the microscopic agglutination test. Live cultures of L. hardjo (Hardjoprojitno) were used as test antigens.

Bacterin potency tests. Two groups of 21 female weanling Syrian hamsters were inoculated I.P. with 10 mg of either the 29° or 37° C grown bacterin. An additional group of 21 hamsters served as a control and was not inoculated with a bacterin. Fourteen days post inoculation all three groups were challenged with 3.0×10^5 cells intraperitoneally of a virulent L. hardjo isolate designated N.Z. #12. Three

animals from each group were euthanized on the following schedule: 2, 6, 12, 24, 48, 96, 144, and 192 hours post challenge.

At necropsy blood, urine, liver, kidney, and brain samples were obtained from each animal. The tissues were placed in 3.0 cc sterile syringes and expressed into 10 ml of 1% BSA. The bladder was emptied of urine and diluted in 10 ml of 1% BSA. A maximum volume of blood was collected by cardiac puncture and 1.0 ml was inoculated into 10 ml of 1% BSA, the remainder was processed for serum.

One ml volumes of BSA containing tissue samples were inoculated into tubes of BA-P80 semisolid media. Blood samples were serially diluted from 10^{-1} to 10^{-10} in 1% BSA then 1.0 ml from each tube was transferred to semisolid. The cultures were incubated at 29° C for 60 days and observed for the appearance of a Dinger's zone.

Titration of virulent L. hardjo challenge. Forty-five female weanling Syrian hamsters were inoculated intraperitoneally with 1.0 ml of culture containing the following cell numbers:

3.0×10^{6} cells - 10 hamsters

3.0×10^{5} cells - 5 hamsters

3.0×10^{4} cells - 5 hamsters

3.0×10^{3} cells - 5 hamsters

3.0×10^{2} cells - 5 hamsters

30 cells - 5 hamsters

3.0 cells - 5 hamsters

0.3 cells - 5 hamsters

The five tissues were cultured from each animal and serum was processed for the determination of antibody titers. Kidneys were also submitted to the Iowa State University Veterinary Diagnostic Laboratory for histopathology interpretation. Infectivity was based upon the re-isolation of viable leptospires from hamster tissues. The hamster infective $dose_{50}$ was calculated using the method of Reed-Meunch.

Hamster passive protection test. Two passive protection tests were run; one used serum from hamsters that were used in the titration of the challenge strain and the second used serum from the hamsters used in the agglutinin production experiment.

Twenty-four female weanling Syrian hamsters were divided into two groups, one of 16 and eight animals respectively. Sixteen hamsters received 1.0 ml of serum from animals that had been inoculated with 3.0×10^6 to 3.0 organisms. Twenty-four hours later they were challenged I.P. with 4.5×10^6 cells of a virulent L. hardjo isolate. The other eight animals were inoculated with 1.0 ml of serum from animals that had received the 29° C bacterin. They were challenged in the same way.

Fourteen days post inoculation the animals were euthanized and blood, urine, liver, kidney, and brain tissues

were obtained. They were cultured in the manner previously described. The cultures were incubated at 29° C for 60 days and observed for macroscopic turbidity.

Microtiter agglutination test. All serum samples obtained in this study were retested using a microtiter procedure (Galton et al. 1965). A plastic disposable plate with 96 flat-bottomed wells (Costar, Cambridge, Massachusetts), a 0.05 ml calibrated diluting loop (Cooke Laboratory Products, Alexandria, Virginia), were used.

The samples were diluted 1:5 in 0.85% phosphate-buffered saline and diluted two-fold from 1:10 to 1:20,480.

Four-day-old antigens adjusted to 25 nephelos were delivered in 0.05 ml volumes by the pipette droppers in each well, making a final volume 0.10 ml. A plastic film cover was placed over the tray during the one-hour incubation period at room temperature.

The test was read in the plate by darkfield examination using a 10X long-working-distance objective (Ernest Leitz, Wetzlar, West Germany). The degrees of agglutination were rated 1+ to 4+, and the end point was the highest dilution demonstrating a 1+ reading.

DATA

Viable cell counts. The cell counts of Leptospira hardjo serotype Hardjoprajitno were based on the mean of ten replicate microscopic fields. A liquid BA-P80 broth culture adjusted to 25 nephelos units yielded an average of 3.4×10^8 per milliliter.

Measurement of growth in liquid media. The growth of Leptospira hardjo was measured using a NADC secondary turbidity standard, rated at 50.5 nephelos, a completely anodized aluminum dry well, and a Coleman 9 nephelocolorimeter.

A comparison of three dry well prototypes revealed that a blackened interior and exterior surface compared most favorable with the standard water well. The anodized well was more durable and did not chip or wear off like the well treated with aluminum ink (see Figure 1).

The comparison of secondary turbidity standards. The secondary standard rated at 50.5 nephelos proved the most all purpose standard (Figure 2).

When the nephelometer readings of liquid cultures were compared to direct microscopic cell counts, the results showed that the standards rated at either the higher or lower nephelos units were not as accurate as the 50.5 standard. Low rated standards would give reduced nephelometer readings when dense cultures were measured, and highly rated

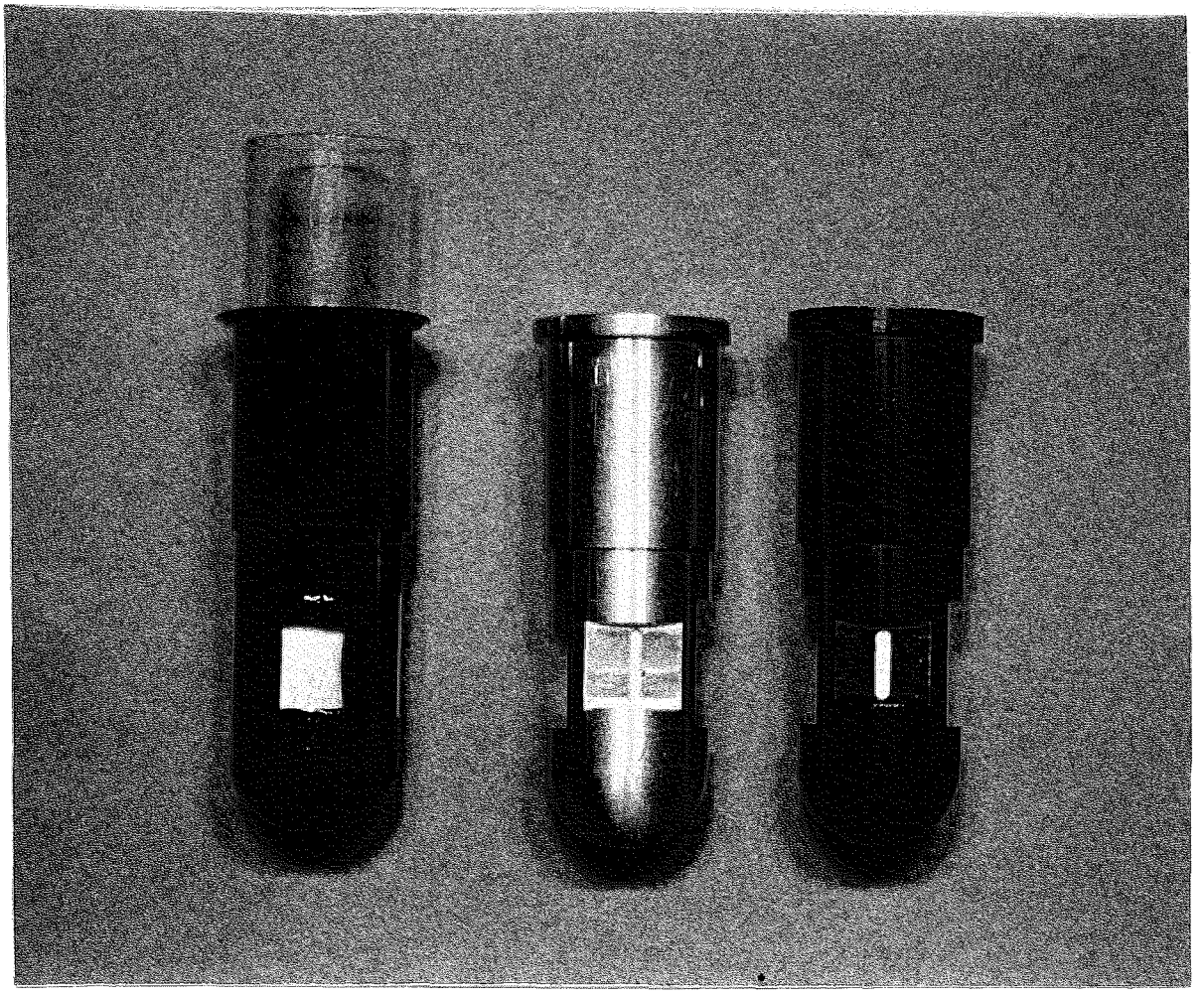

Figure 1. A comparison of three nephelometer cuvettes evaluated in this study. The well on the left is the water well supplied by the manufacturer, the center well is an experimental aluminum dry well cuvette. The cuvette on the right is identical to the aluminum prototype except that the exterior has been anodized.

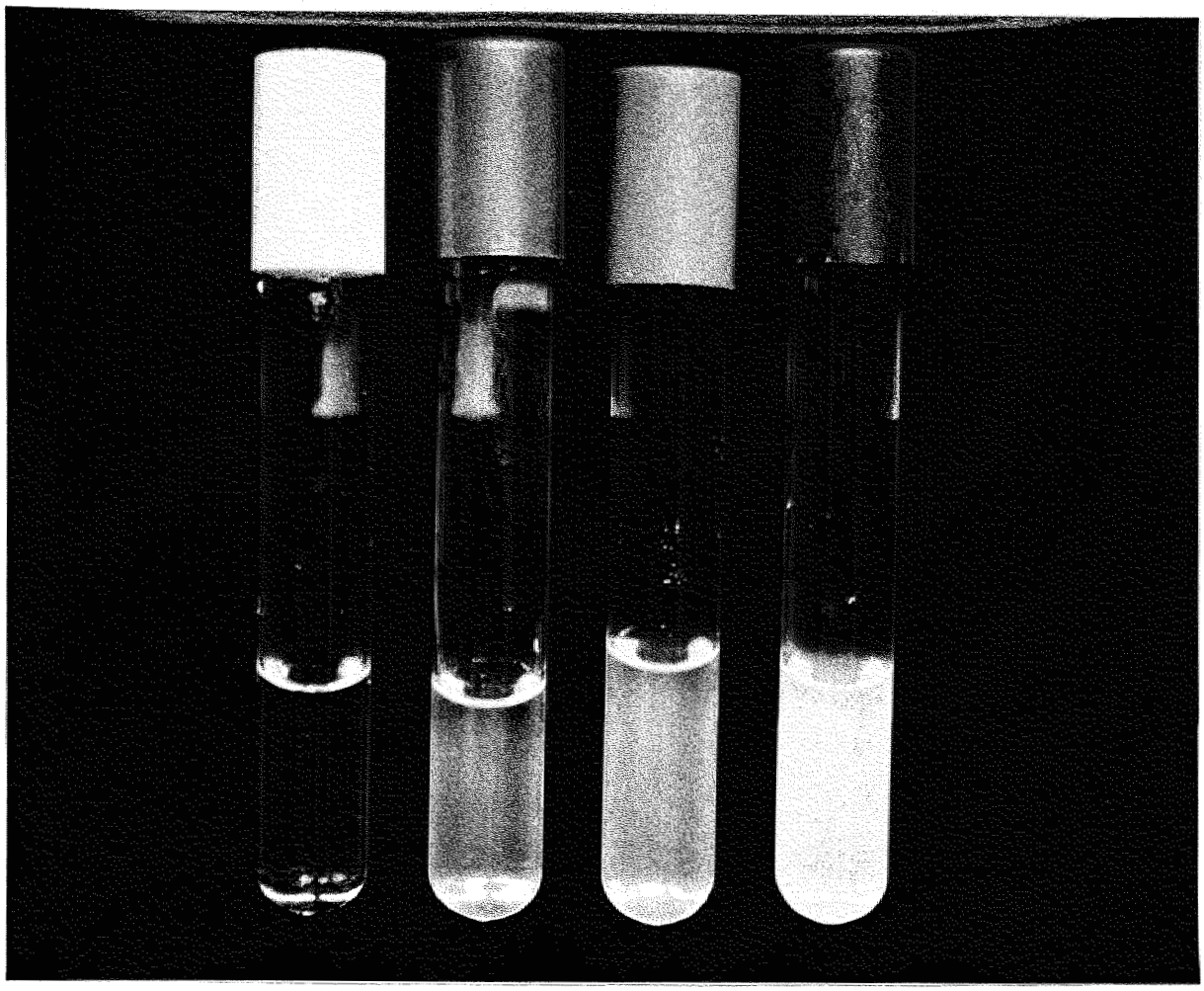

Figure 2. The four NADC secondary nephelometer standards used for the measurement of the growth of leptospira in liquid culture. The white capped standard was rated at 0 nephelos, the green one at 31.5 nephelos, the blue one at 50.5 nephelos, and the red capped tube at 115 nephelos units.

standards would give higher turbidity readings when diluted cultures were used. The secondary standard rated at 50.5 nephelos showed less variance across the total turbidity scale than either of the other two standards. Also, it was more practical to select one standard to be used for all experiments than to rely on several standards that required readjustment of the nephelocolorimeter (Figure 3).

Scale expansion using secondary standards. The use of scale expansion was useful in detecting small changes in turbidity values. By adjusting the machine to twice the rated value of the standard, the turbidity reading of the culture was also increased two-fold, thereby increasing the sensitivity of the nephelometer. Also in the case of dense turbidity the instrument was adjusted to one-half of the rated value of the culture which would allow a reading on the nephelos scale. A comparison of growth curves using scale expansion is shown in Figure 4.

Nephelometry for the measurement of the growth of liquid cultures. Nephelometer values were greater than the % T readings when the growth of a liquid media culture was measured. Figure 5 illustrates how the albumin enriched media absorbs the three different wavelengths of light. Because nephelometry does not measure either absorbed or transmitted light any changes in measured density were due to an increase in the total cellular mass of the culture.

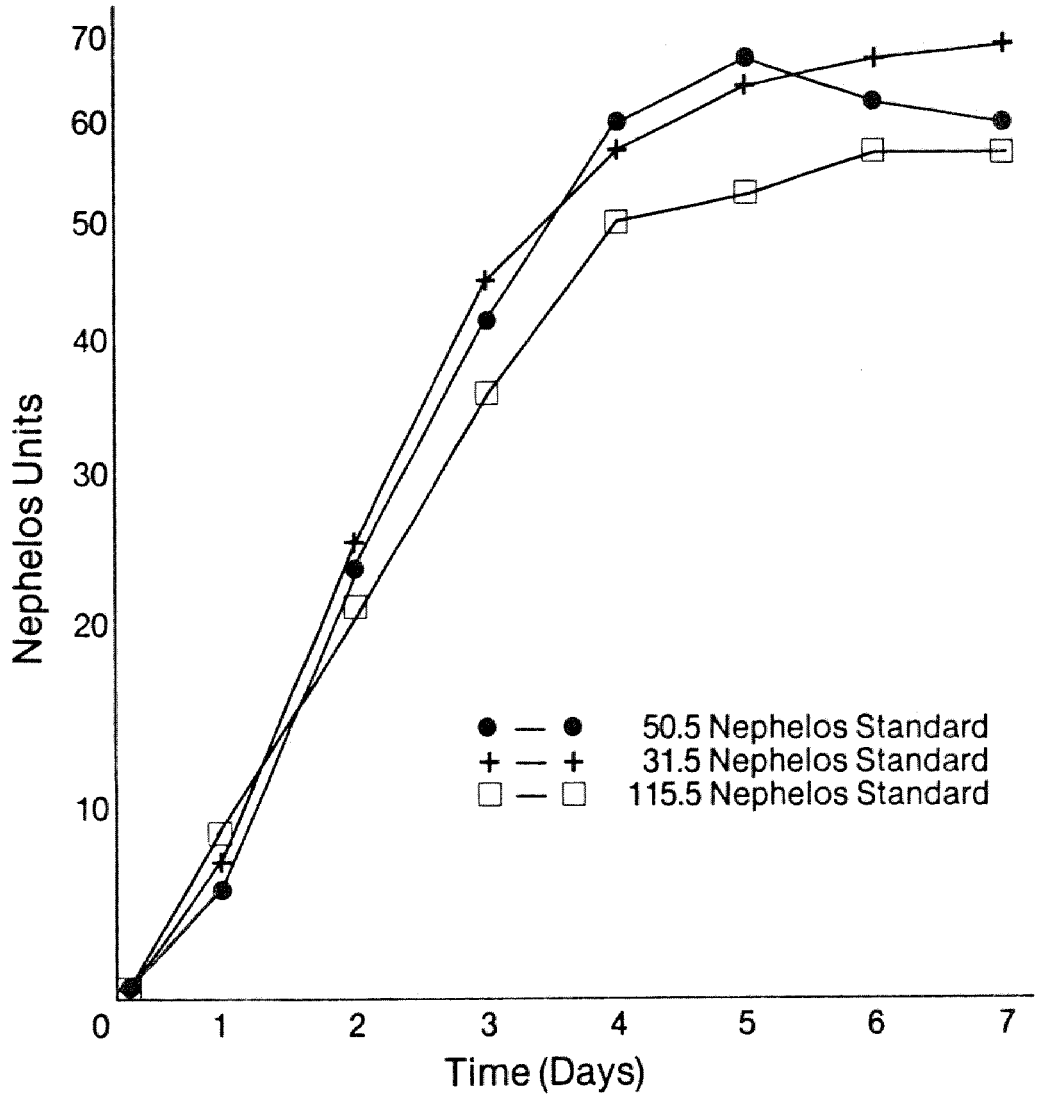

Figure 3. The effect of various turbidity standards upon growth measurement of a single liquid culture of L. hardjo. The Coleman 9 nephelometer was adjusted to each standard and used to monitor a single tube of BA-P80 media incubated at 29° C over seven days.

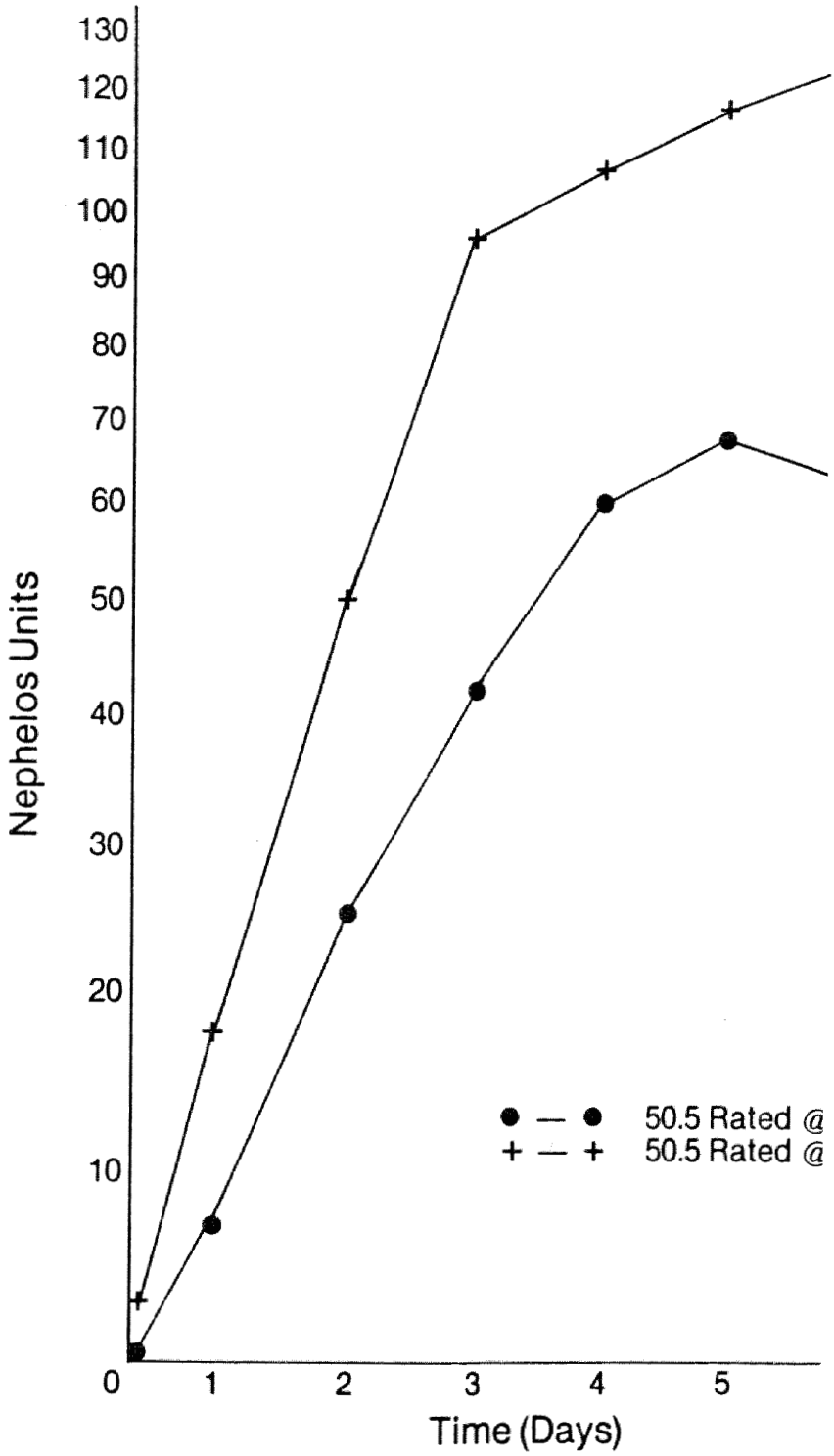

Figure 4. The effect of adjusting a Cole colorimeter to twice the rated value of the NA Growth of a liquid L. hardjo culture inoculate organisms/ml and incubated at 29° C was monito 50.5 nephelos standard and with the instrument 101 nephelos.

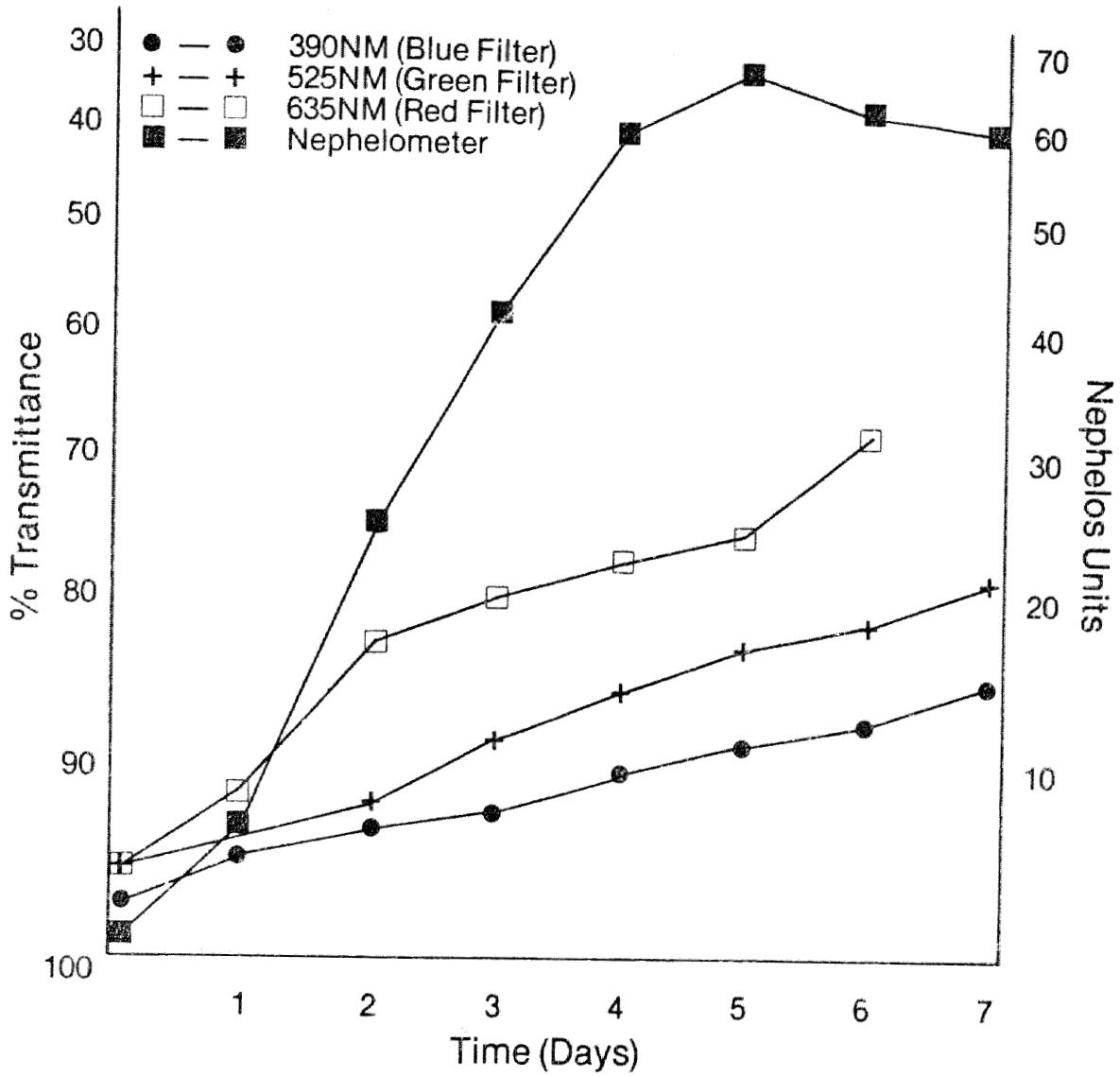

Figure 5. A comparison of the sensitivity of nephelometry versus spectroscopy at three different wavelengths using a Coleman 9 nephelocolorimeter. A single liquid culture of L. hardjo was inoculated with 1.0 ml of inoculum standardized to 3.4 x 10^8 cells/ml and was incubated at 29° C for nine days. A nephelometric standard rated at 50.5 nephelos was used throughout.

Since nephelometry ignores the aspects related to color, it was selected as the method of measuring turbidity.

Growth of large cell numbers in substituted and deleted liquid media. An inoculum of 3.4×10^8 cells/ml of L. hardjo showed very little difference in the ability to grow in various polysorbate supplemented media. Tween 80 and 60 were preferred to Tween 40 and 20. A comparison of growth is shown in Figure 6.

A single deletion of polysorbate 80, NH_4Cl, L-cystine, and vitamins B_1 and B_{12} reduced the growth pattern. The initial deletion did not drastically reduce growth. The growth in the absence of polysorbate, NH_4Cl, and L-cystine is given in Figure 7, and the effect of vitamin deletion in Figure 8.

Multiple deletions of trace metals or the trace metals and L-cystine had little effect on total growth. The nutritive value of bovine serum albumin was evaluated by deleting L-cystine from media devoid of NH_4Cl and polysorbate 80. Total growth was reduced compared to complete medium, but similar to media deleted only of the polysorbate fraction (Figure 8).

When the cells were washed prior to subculturing the nutritional effects of the various media were magnified. Figures 9, 10 and 11 illustrate this fact.

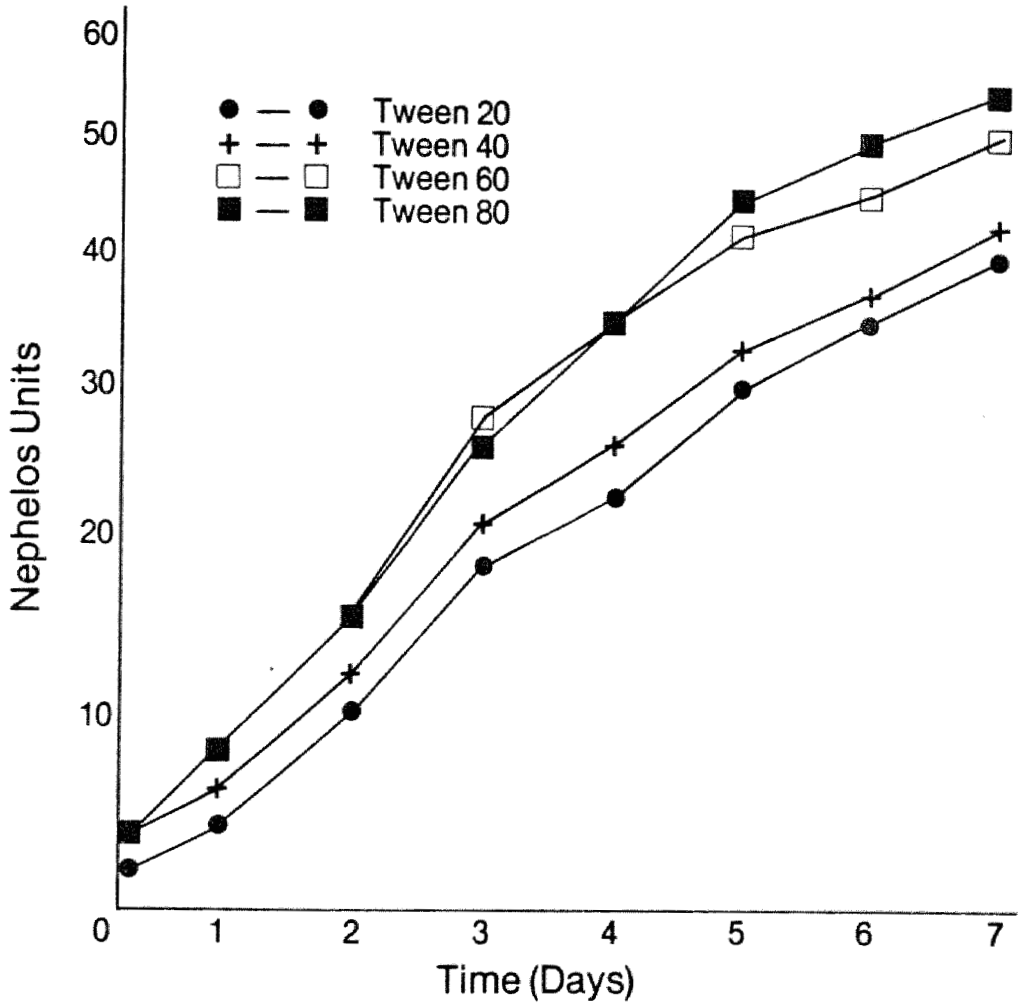

Figure 6. The effect on total growth of L. hardjo when bovine albumin polysorbate media was supplemented with one of four Tweens. The cultures were each inoculated with $3.4x10^8$ cells per ml and incubated statically at 29° C for one week.

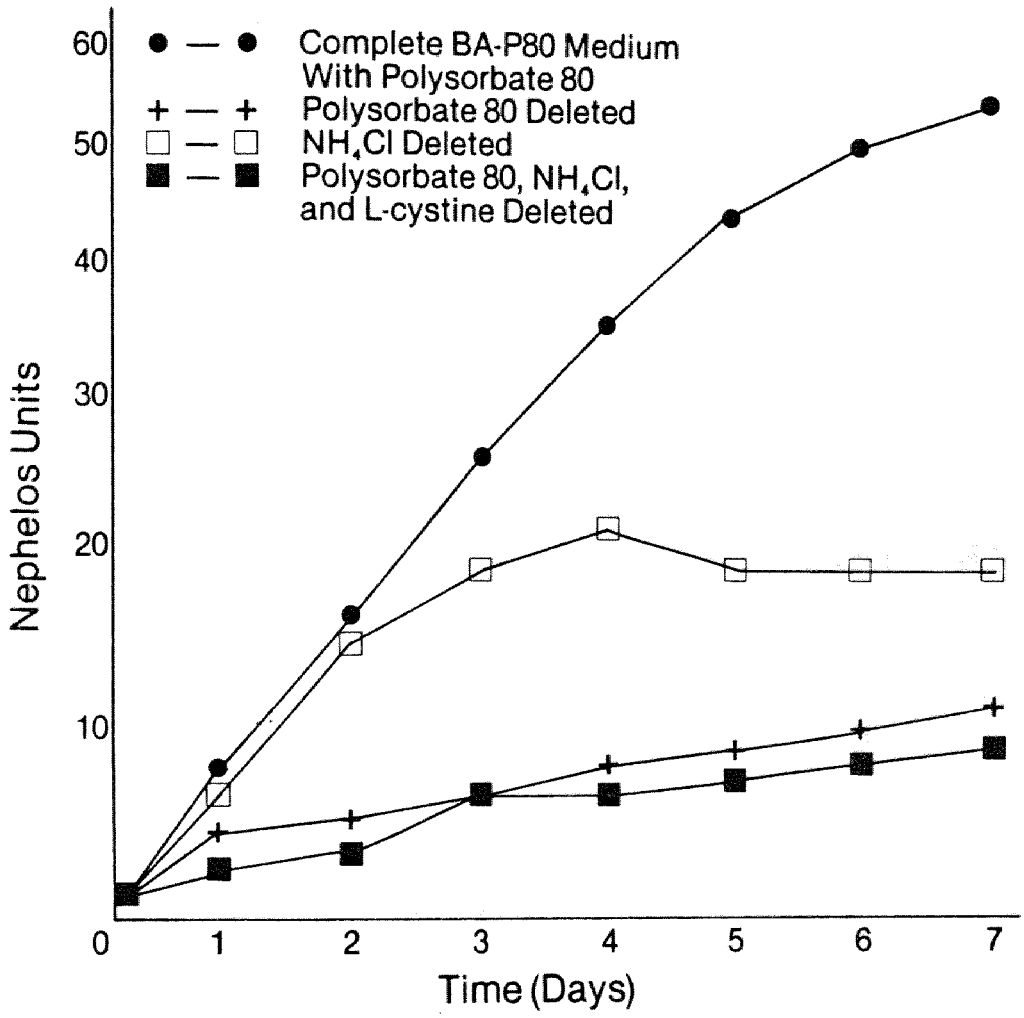

Figure 7. The decrease in total measurable growth when deletions of polysorbate 80, NH_4Cl, and L-cystine were made from BA-P80 media. The cultures were inoculated with $3.4x10^8$ cells/ml and incubated at 29° C for seven days.

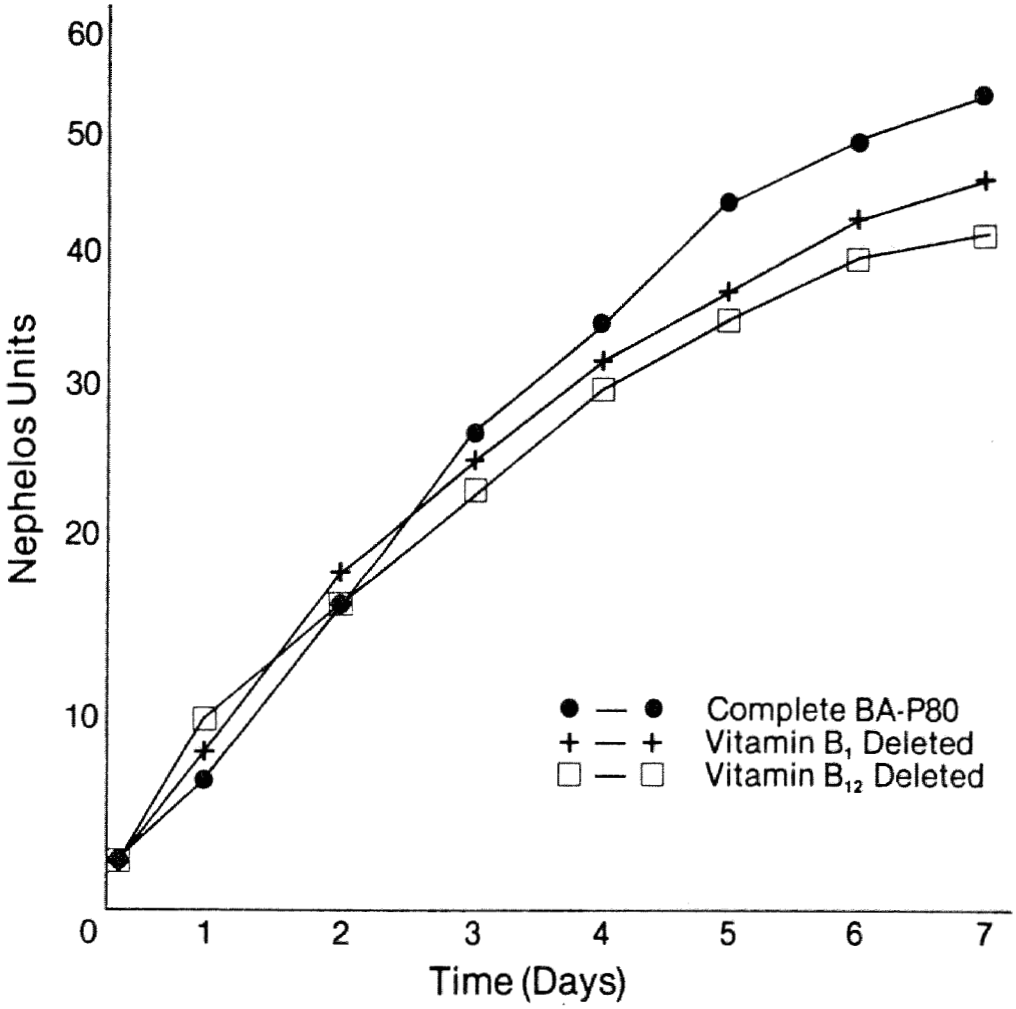

Figure 8. The effect of the single deletion of either vitamin B_1 or B_{12} as compared to total growth in complete BA-P80 media. The media was inoculated with 1.0 ml containing $3.4x10^8$ leptospires/ml. The cultures were incubated at 29° C for seven days.

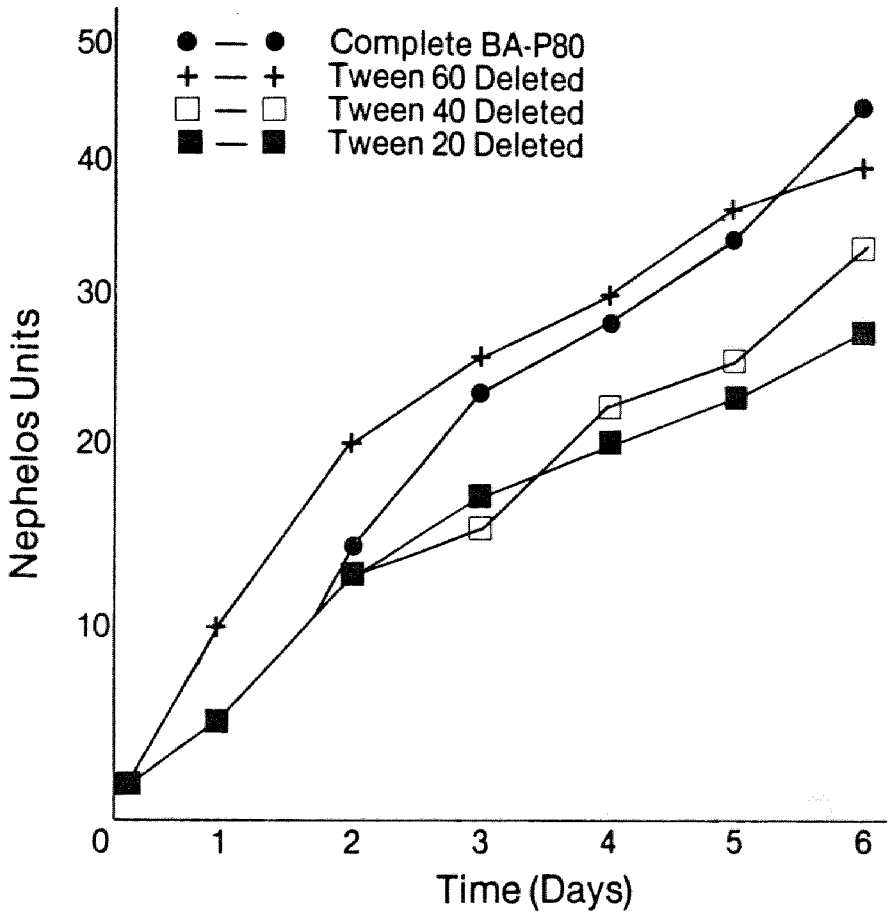

Figure 9. The effect of the deletion of the polysorbate fraction from BA-P80 media upon total growth at 29° C. Each culture was inoculated with $3.4x10^7$ cells that were washed three times in 0.005M sterile phosphate buffer before inoculation.

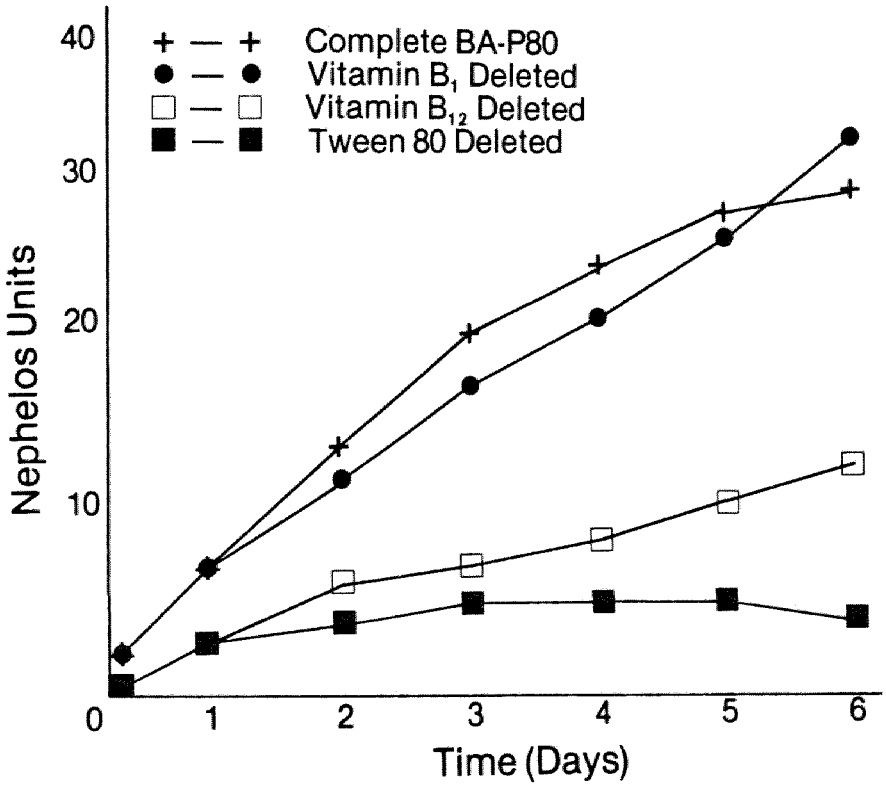

Figure 10. The effect of deletion of polysorbate 80, vitamin B_1 and vitamin B_{12} compared to the growth of L. hardjo in complete BA-P80 media incubated at 29° C. The inoculum was washed three times in 0.005M phosphate buffer before use.

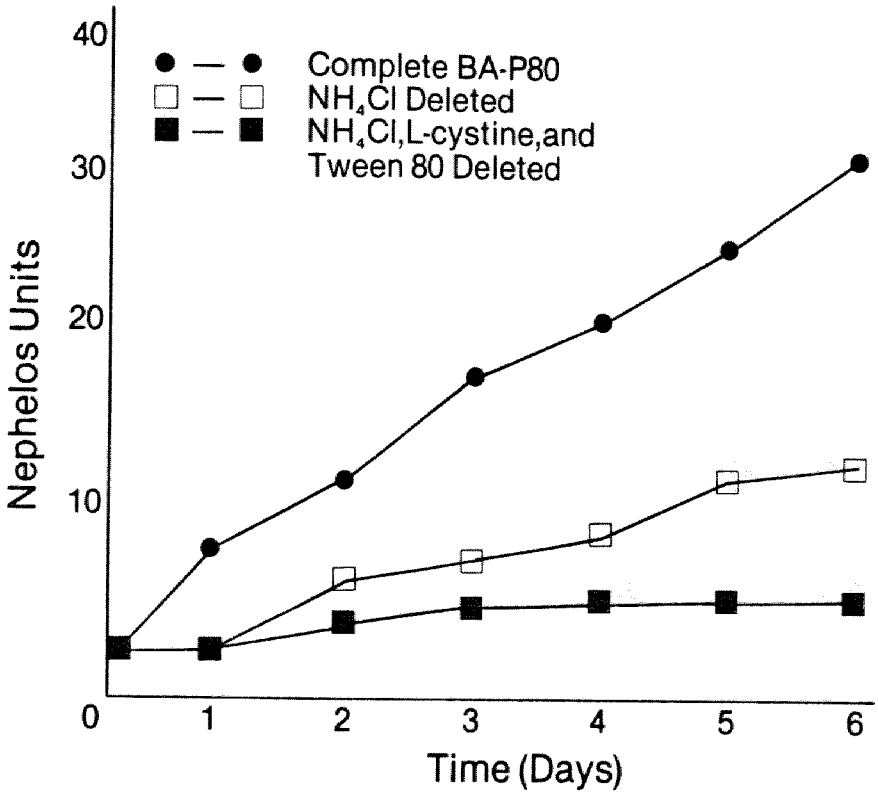

Figure 11. A comparison of the growth of washed cells of L. hardjo in liquid media deleted of: NH_4Cl; NH_4Cl, L-cystine, and Tween 80; or complete BA-P80 media. The cells were washed in 0.005M phosphate buffer.

Dilution extinction studies in deleted and substituted liquid BA-P80 media. Tables 3, 4 and 5 show the maximum nephelometer readings of growth in serial dilution after 7, 14, and 21 days. These values demonstrate the effects of different nutritional environments on total growth as well as the ability of the media to support the growth of small cell numbers.

The ability of the four Tweens to support the growth of a small inoculum is comparable based on macroscopic turbidity. Only Tween 40 was unable to support the growth of an inoculum less than 3 cells/ml.

The initial single deletion of polysorbate 80, NH_4Cl, Vitamin B_1, and L-cystine did not drastically reduce growth of minimal cell numbers. However, the deletion of Vitamin B_{12} results in no growth after a 10^{-3} dilution.

The deletion of trace metals and trace metals plus L-cystine did not greatly reduce growth. The triple deletion of polysorbate, NH_4Cl, and L-cystine drastically reduced growth when the inoculum was diluted more than 1:100.

Growth in liquid media using continuous subculture. Polysorbate 80, 60, 40, and 20 all were similar in their ability to support the growth of L. hardjo through five continuous subcultures. When the Tween 80 fraction was deleted from the media, the turbidity was drastically reduced (Table 6).

The single deletion of vitamins B_1, B_{12}, or NH_4Cl also

Table 3

Growth of Fewest Cell Numbers in Deleted Liquid Medium after Seven Days*

Media	Inocula: Number of cells per ml			
	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$
Polysorbate 80	24	4	2	0
Polysorbate 60	38	28	12	4
Polysorbate 40	27	19	9	3
Polysorbate 20	17	11	6	2
Polysorbate 80 deleted	8	8	7	5
NH_4Cl deleted	16	16	13	5
Vitamin B_1 deleted	16	10	4	0
Vitamin B_{12} deleted	14	5	3	0
L-cystine deleted	33	26	18	7
Trace metals deleted	40	30	6	0
Trace metals and L-cystine deleted	35	27	6	0
Tween 80, NH_4Cl, and L-cystine deleted	7	4	0	0
Ellinghausen-McCollough-Johnson-Harris (EMJH) media	22	1	0	0
Stuart's	5	5	0	0
Oleic-albumin complex	26	1	0	0
Asbestos-filtered BA-P80	13	12	0	0

*The figures represent the maximum turbidimetric value of cultures inoculated with L. hardjo 7 days after inoculation. The cultures were incubated as static cultures of 29° C in bovine albumin polysorbate basal media formulations as indicated.

Table 4

Growth of Fewest Cell Numbers in Deleted Liquid Medium After 14 Days*

Media	Inocula: Number of cells per ml						
	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$
Polysorbate 80	44	48	52	43	36	24	6
Polysorbate 60	45	52	48	41	34	20	4
Polysorbate 40	41	44	38	31	23	12	4
Polysorbate 20	52	37	27	14	9	0	0
Polysorbate 80 deleted	6	4	4	4	0	0	0
NH_4Cl deleted	11	11	10	11	12	12	0
Vitamin B_1 deleted	15	8	8	0	0	0	0
Vitamin B_{12} deleted	21	3	0	0	0	0	0
L-cystine deleted	37	35	38	38	34	29	15
Trace metals deleted	53	56	44	30	13	0	0
Trace metals and L-cystine deleted	48	47	50	41	33	26	13
Tween 80, NH_4Cl, and L-cystine deleted	5	2	2	0	0	0	0
Ellinghausen-McCollough-Johnson-Harris (EMJH) media	39	22	0	0	0	0	0
Stuart's	4	3	3	0	0	0	0
Oleic-albumin complex	36	31	2	0	0	0	0
Asbestos-filtered BA-P80	11	0	0	0	0	0	0

*The maximum turbidity values of static cultures of L. hardjo fourteen days after inoculation. All of the static cultures were incubated at 29° C.

Table 5

Growth of Fewest Cell Numbers in Deleted Liquid Medium After 21 Days*

Media	Inocula: Number of cells per ml							
	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
Polysorbate 80	40	45	45	53	56	53	56	51
Polysorbate 60	38	41	47	46	44	55	47	31
Polysorbate 40	33	38	38	41	51	48	44	28
Polysorbate 20	41	35	44	47	42	30	21	0
Polysorbate 80 deleted	5	6	5	4	5	4	5	0
NH_4Cl deleted	11½	12	10	10	10	11	12	0
Vitamin B_1 deleted	18	8	6	8	8	8	0	0
Vitamin B_{12} deleted	22	4	3	0	0	0	0	0
L-cystine deleted	41	36	38	41	41	44	34	0
Trace metals deleted	43	45	45	55	54	39	34	0
Trace metals and L-cystine deleted	46	47	54	46	48	55	53	48
Tween 80, NH_4Cl, and L-cystine deleted	4	2	0	0	0	0	0	0
Ellinghausen-McCollough-Johnson-Harris (EMJH) media	35	26	0	0	0	0	0	0
Stuart's	2	0	0	0	0	0	0	0
Oleic-albumin complex	30	30	0	0	0	0	0	0
Asbestos-filtered BA-P80	10	0	0	0	0	0	0	0

*The turbidity measurements of static cultures incubated 21 days after inoculation with L. hardjo. The cultures were incubated at 29° C during the 3 week period.

Table 6

The Effect of Deleted Medium on Five Continuous Subcultures*

Media	Number of weekly continuous subcultures				
	1	2	3	4	5
Polysorbate 80	54	58	52	56	55
Polysorbate 60	42	52	58	54	43
Polysorbate 40	32	48	47	46	39
Polysorbate 20	34	37	37	40	40
Polysorbate 80 deleted	5	5	5	6	4
NH_4Cl deleted	15	13	7	14	6
Vitamin B_1 deleted	6	7	14	19	15
Vitamin B_{12} deleted	4	1	2	1	1
L-cystine deleted	47	61	61	58	56
Trace metals deleted	49	60	63	60	58
Trace metals and L-cystine deleted	50	55	58	47	46
Tween 80, NH_4Cl, and L-cystine deleted	9	4	2	2	4
Ellinghausen-McCollough-Johnson-Harris (EMJH) media	23	30	0	0	0
Stuart's	0	2	0	0	0
Oleic-albumin complex	23	25	28	18	19
Asbestos-filtered BA-P80	16	16	15	17	16

*The figures represent the maximum nephelometer reading attained by static cultures of L. hardjo inoculated with 3.4×10^8 cells/ml and held at 29° C. Weekly subcultures were made by diluting the inoculum of 0.005M phosphate buffer in order to readjust the inoculum to a turbidity of 25 nephelos. The next successive culture received 1 ml of this washed cellular preparation which also contains approximately 3.4×10^8 cells/ml.

reduced the measurable growth. Each successive subculture resulted in lower total growth except in the case of vitamin B_1 where it appears that some adaptation occurs. The deletion of L-cystine or the trace metals (Fe^{+2}, Cu^{+2}, Zn^{+2}) singly did not seem to affect the growth of the leptospires. However, when Tween 80, NH_4Cl, and the L-cystine were diluted at the same time the measurable growth was minimal.

The Ellinghausen-McCollough-Johnson-Harris modified formulation only passed twice before the nephelometer readings dropped to zero. The Stuart's media reached two nephelometer units on the second subculture, but the other passages read zero. The oleic acid complex and Stuart's formulations supported low growth for five subcultures.

The polysorbate 80, NH_4Cl, L-cystine, and the triple deletion media allowed a low level of growth when inoculated with $3.4x10^8$ cells. When vitamin B_1 was omitted, the media maintained the viability of the initial inocula approximately $3.4x10^8$ cells/ml, but did not stimulate growth. When vitamin B_{12} was left out of the media the culture did not grow in the second subculture.

Dilution extinction growth studies in deleted and substituted BA-P80 semisolid media. The growth pattern of L. hardjo in semisolid media resembled the growth in liquid media. No attempt was made to quantitate the cellular mass, instead the cultures were examined macroscopically every seven days for four weeks for evidence of growth.

Polysorbate 80 complete basal media was the best medium for the growth of minimal cell numbers. Tween 40 or 60 could serve as substitutes for polysorbate 80 but Tween 20 could not support the growth of inocula less than $3.4x10^4$ cells (Table 7). The effect of single deletion can be seen in Table 7. The ability to support the growth of small cell numbers was limited in these preparations.

Simple semisolid media were evaluated in order to emphasize the importance of the bovine serum albumin fraction and vitamin B_1 and B_{12}. Growth was observed microscopically when the concentration of the inocula was greater than $3.4x10^5$ cells/ml in media containing agar, albumin, and vitamin B_{12}; agar, albumin, vitamins B_1 and B_{12}, and in the 0.1% agarose and phosphate buffer media. Maximum growth occurred at $3.4x10^5$ cells/ml or less in the remaining media. The formulation containing only agar and phosphate buffer showed no evidence of growth, while the addition of 0.1% or 0.2% agarose supported limited growth as seen in Table 7.

Evaluation of agar products in semisolid media. The growth of minimal cell numbers in various agar samples is shown on Table 8. This type of assay proved valuable in selecting agar for incorporation in isolation semisolid media. Eight agars were rated as superior based on the ability to support the growth of 34 cells or less within four weeks post inoculation. The growth was also

Table 7

Growth of Fewest Cell Numbers in Deleted Semisolid Medium**

Semisolid	Cells per milliliter								
	$3.4x10^8$	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
Polysorbate 80	7	7	7	12	12	12	21	21	21
Polysorbate 60	7	7	7	12	21	21	21*	0	0
Polysorbate 40	7	7	7	12	12	21	21	21	0
Polysorbate 20	7	12	12	21*	21*	0	0	0	0
Polysorbate 80 deleted	7	7	12	21*	21*	21*	0	0	0
NH_4Cl deleted	7	7	7	14	14	14	14	21*	0
Vitamin B_1 deleted	7	7*	7*	14	14	14	21*	21*	0
Vitamin B_{12} deleted	7	7*	7*	21*	21*	21*	21*	0	0
Agar + PO_4	0	0	0	0	0	0	0	0	0
Agar and Albumin	21*	21*	21*	0	0	0	0	0	0
Agar, Albumin, Vitamin B_1	7	21*	21*	21	0	0	0	0	0
Agar, Albumin, Vitamin B_{12}	7*	21*	21*	21*	0	0	0	0	0
Agarose 0.1%	21*	21*	21*	21*	0	0	0	0	0
Agarose 0.2%	21*	21*	21*	0	0	0	0	0	0

**The growth of minimal cell numbers of L. hardjo in nutritionally deleted semisolid media. A standardized inoculum of $3.4x10^8$ cells/ml was diluted serially ten-fold in sterile 1% bovine serum albumin diluent. The tubes were examined macroscopically every 7 days for 3 weeks. The figures represent the first time that visible growth was recorded. All tubes exhibiting signs of no growth were examined microscopically with a darkfield condenser and are marked with an asterisk if the culture was found to contain viable organisms. Zeros indicated cultures in which viable leptospires were not observed.

Table 8

The Effect of Various Grades of Agar on the Growth of Fewest Cell Numbers in Semisolid Medium*

	Inoculum: cells per milliliter								
Agar Source	$3.4x10^8$	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
BBL 412601	7	7	7	14	14	21	0	0	0
Fisher 735208	7	7	7	0	0	0	0	0	0
BBL JGDCHR	7	7	7	7	14	14	14	0	0
MEER UC3652	7	7	7	7	0	0	0	0	0
FLAKE 6116815	7	7	7	14	14	14	14	14	0
Inolex 26-7	7	7	7	14	0	0	0	0	0
NBC 4332	7	7	7	7	14	21	28	0	0
BBL 3076095	7	7	7	7	14	14	21	28	0
BBL 0116305	7	7	7	14	14	21	21	21	0
TECH 504380	7	7	7	14	7	0	0	0	0
BACTO 6098945	7	7	7	7	14	14	21	14	0
Noble 6122775	7	7	7	7	14	14	14	21	0
Noble 612532	7	7	7	7	14	14	28	0	0
Noble 577089	7	7	7	7	14	14	21	21	0
Noble 504461	7	7	7	7	14	14	21	0	0
P504461	7	7	7	14	14	14	28	28	0
P504274	7	7	7	14	0	21	28	0	0
FLAKE 609714	7	7	7	7	7	14	14	0	0

All tubes that were macroscopically negative were examined microscopically 21 days post incubation. The number represents the day that macroscopic growth was visible. The zeros indicate an absence of viable bacteria.

macroscopically detectable and eliminated the use of direct microscopic examination of the cultures. The Flake agar lot #611681 and the Bacto lot #609894 were the best samples because they supported the growth of an inoculum of 34 cells and the growth was detectable within two weeks following inoculation of the media.

Seven agars were rated as average when they were shown to be capable of supporting the growth of at least 34,000 cells. Three agars were judged of inferior quality because they would not maintain the viability of at least 340,000 cells.

Evaluation of albumin samples in liquid BA-P80 medium. Eighteen albumin samples were compared to the Miles bsa reference lot #231 for their ability to stimulate maximum growth of L. hardjo as measured by nephelometry. Table 9 illustrates the nephelometer readings of the cultures over seven days incubation at 29° C. The Gibco albumin sample and the Phillips-Roxanne sample surpassed the 71 nephelos reading of the reference albumin. The turbidity due to leptospiral growth exceeded 65 nephelos units in nine albumin samples. The rabbit and porcine albumin samples were average in their growth supporting ability, while the equine albumin and three Wilson product samples were rated as unsatisfactory because of their lower nephelometer readings.

When serial ten-fold dilutions of the L. hardjo inoculum were made, the growth promoting qualities of the

Table 9

Growth of L. hardjo in Liquid Medium Containing Various Albumins*

Albumin Type	Day:	Daily Nephelometer Reading 0	1	2	3	4	5	6	7
Fatty Acid poor lot #7		2	6	15	31	49	62	67	67
Crystalline lot #23		3	7	17	23	37	60	60	59
Miles pH 7.2		2	8	23	39	56	59	67	65
Miles pH 7.4		2	8	23	38	56	62	68	65
Miles pH 7.6		2	9	25	38	52	61	66	63
Miles pH 7.8		2	7	28	40	52	58	64	63
Rabbit		2	7	26	35	44	51	57	58
Porcine		2	7	22	37	54	60	64	64
Equine		2	6	11	15	20	25	30	32
Miles lot #29		2	7	21	35	49	51	65	64
Miles lot #163		2	7	23	39	57	63	70	67
Fatty acid free lot #163		2	8	24	38	55	63	68	65
Reheis bovine		2	7	23	35	47	53	64	72
Gibco bovine		3	6	11	17	28	57	87	87
Wilson lot #1		4	4	4	5	6	8	9	10
Wilson lot #2		4	3	4	5	6	8	10	12
Wilson lot #3		4	3	4	5	5	7	7	9
Phillips-Roxanne		1	5	14	26	36	59	79	86
NADC lot #231 control		2	6	19	36	54	63	71	69

*The daily growth of L. hardjo in bovine albumin polysorbate 80 liquid media supplemented with different albumin. The tubes were all inoculated with 3.4×10^7 cells per 10 ml tube and incubated as static cultures at 29° C.

albumins was intensified. Table 10 shows the seven day post inoculation nephelometer figures. The majority of the albumins performed the same at the first dilution, but the differences in growth supporting capabilities was evident by the third dilution. After three weeks incubation, eight albumin products were capable of supporting the growth of an inoculum theoretically adjusted to approximately three cells, but the nephelometer readings were less than those observed with higher inoculum concentrations (Table 11).

Interaction of agar and bovine serum albumin upon growth. When albumins and agar judged of superior and inferior quality were combined in several ways, the ability to promote growth varied (Table 12). When the reference albumin Miles lot 231 was added to semisolid media made with Fisher agar lot #735208, growth was detected with an inoculum of approximately three cells. But when a semisolid medium was made with Fisher agar and a Wilson albumin sample, the inoculum was only viable at a concentration of 3.4×10^7 cells per tube. Fisher agar without albumin supplementation maintained the viability of an inoculum of 3.4×10^7 cells. The BBL agar lot #011630 was able to support the viability of 3.4×10^6 cells, which was equal to the performance of the same agar supplemented with Gibco bovine albumin.

Table 10

The Effect of Various Albumins on the Growth of Fewest Cell Numbers After Seven Days*

Albumin Type	Inocula: (cells per tube) $3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$
Fatty Acid Poor lot #7	62	60	2	0
Crystalline lot #23	60	60	3	1
Miles pH 7.2	59	62	17	4
Miles pH 7.4	62	63	15	4
Miles pH 7.6	61	60	16	4
Miles pH 7.8	58	62	13	2
Rabbit	51	50	14	2
Porcine	60	61	10	1
Equine	25	2	0	0
Miles lot #29	58	64	10	1
Miles lot #163	63	65	14	4
Fatty Acid Free lot #163	63	61	14	1
Reheis bovine	53	54	17	1
Gibco bovine	57	7	0	0
Wilson lot #1	8	1	0	0
Wilson lot #2	8	1	0	0
Wilson lot #3	7	1	0	0
Phillips-Roxanne	59	70	3	0
NADC lot #231 control	63	65	0	0

*The seven day post-inoculation nephelometer readings of L. hardjo in various albumins added to BA-P80 media.

Table 11

The Effect of Various Albumins on the Growth of Fewest Cell Numbers after 21 Days

Albumin Type	Inocula: (cells per tube) $3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
Fatty Acid Poor lot #7	42	43	50	55	51	38	30	11
Crystalline lot #23	31	55	53	60	56	42	32	10
Miles pH 7.2	40	40	50	47	47	46	44	0
Miles pH 7.4	41	39	30	53	51	45	48	0
Miles pH 7.6	41	35	45	48	47	45	40	0
Miles pH 7.8	41	30	41	52	52	48	35	0
Rabbit	45	44	43	41	40	38	35	28
Porcine	41	39	47	44	46	40	42	20
Equine	43	1	0	0	0	0	0	0
Miles lot #29	46	43	51	58	53	48	22	2
Miles lot #163	50	53	55	56	56	52	49	44
Fatty Acid Free lot #163	49	48	51	52	50	45	47	44
Reheis bovine	44	36	50	50	54	47	42	32
Gibco bovine	73	57	10	0	0	0	0	0
Wilson lot #1	33	2	0	0	0	0	0	0
Wilson lot #2	31	4	0	0	0	0	0	0
Wilson lot #3	29	4	0	0	0	0	0	0
Phillips-Roxanne	57	70	43	50	40	26	4	0
NADC lot #231 control	52	55	+	+	+	+	+	40

+ signs indicate viable leptospires observed in cultures contaminated with other bacteria.

Table 12

The Effect of Various Agar and Albumin Samples on the Growth of Fewest Cell Numbers

Media	Inoculum: Number of cells per tube								
	$3.4x10^8$	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
Fisher Agar #735208 + Miles BSA #231	+	+	+	+	+	+	+	+	+
Fisher Agar #735208 + Wilson BSA	+	+	-	-	-	-	-	-	-
Fisher Agar #735208 alone	+	+	-	-	-	-	-	-	-
BBL Agar #011630 + Miles BSA #231	+	+	+	+	+	+	+	+	+
BBL Agar #011630 + Gibco BSA	+	+	+	-	-	-	-	-	-
BBL Agar #011630 alone	+	+	+	-	-	-	-	-	-

Plus signs indicate viable leptospires. Minus signs indicate no viable leptospires observed.

The growth of L. hardjo in gamma irradiated albumin. The treatment of the albumin with gamma irradiation did not prove detrimental to its ability to support the growth of L. hardjo. When the two irradiated samples were compared to two untreated samples of the same lot 261, the difference between the four was only five nephelos units. However, throughout the study, the albumin treated with 2.5 megarads consistently had the lowest maximum nephelos readings (Table 13).

The effect of various concentrations of BSA on growth. The results of the growth of L. hardjo in different concentrations of bovine albumin is summarized on Table 14. All of the concentrations including 0 percent albumin were capable of growing L. hardjo, but growth was reduced at final concentrations less than 0.15% in the first subculture. Albumin concentrations in excess of 3% also reduced the total growth in liquid BA-P80 media.

The maximum growth was seen in media containing concentrations of albumin of 0.4% up to 2.0%. The 2.0% media containing albumin demonstrated reduced maximum turbidity readings after the third subculture.

By the fifth continuous subculture, the media containing 0.0 percent albumin could still support growth that could be measured by the nephelometer, and direct darkfield examination of the culture confirmed that the leptospires were still viable.

Table 13

The Growth of L. hardjo in Bovine Serum Albumin Fraction V Sterilized by Gamma Irradiation*

	Inoculum: cells per ml								
Bovine Albumin	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	340	34	3.4
NZ Lot 261	63	60	59	55	56	56	55	55	47
NZ Lot 261 - 1¼ megarads	58	59	53	56	55	55	55	58	51
NZ Lot 261 - 2½ megarads	56	53	51	56	59	57	55	52	35
A-13 Lot 261	58	57	57	56	59	63	65	59	60

*The maximum turbidity readings of L. hardjo cultures supplemented with New Zealand bovine albumin Lot 261, Lot 261 treated with gamma irradiation, or a second untreated portion of Lot 261 supplied by the manufacturer. The figures reported are in nephelos units using a Coleman 9 nephelo colorimeter and NADC secondary standard.

Table 14

The Effect of Various Concentrations of Albumin on the Growth of Five Continuous Subcultures*

Percent Bovine Albumin	Maximum Nephelometer Reading Subcultures: (7 days) 1	2	3	4	5
0.00	22	26	26	31	26
0.05	16	29	26	28	25
0.10	13	34	33	32	32
0.15	54	46	47	32	35
0.20	50	48	34	34	43
0.40	51	50	53	56	41
0.60	53	53	51	55	41
0.80	54	50	52	49	47
1.00	56	48	51	55	46
2.00	53	51	58	50	44
3.00	42	37	51	48	41
4.00	39	56	41	42	35
5.00	25	32	37	35	33

*The cultures were subcultured at seven-day intervals. The first subculture was inoculated with 3.4×10^8 cells/ml, while all successive subcultures were inoculated with 1 ml from the previous subculture. All liquid cultures were incubated at 29°C.

The effect of temperature on growth. A comparison of the maximum growth of L. hardjo in liquid BA-P80 media at incubation temperatures ranging from 7° C to 40° C is seen in Figures 12 through 14. Organisms incubated at 7° C or 13° C for 28 days showed no measurable increase in turbidity as measured by the nephelometer. However, when a sample of the culture was inoculated into BA-P80 semisolid and incubated at 29° C, viable organisms were isolated. The first temperatures at which a continuous increase in turbidity was recorded occurred at temperatures of 14° C and above.

As the incubation temperature was increased, the lag time of the growth curve was reduced and the maximum turbidity reading was reached in less time. However, at temperatures in excess of 31.5° C, the maximum nephelometer reading declined as the incubation temperatures increased. At a temperature of 40° C and higher steadily increasing turbidity was not evident in liquid BA-P80 media. However, when L. hardjo was inoculated into BA-P80 semisolid media, growth of a large cell inocula was possible at this temperature.

When serial ten-fold dilutions of L. hardjo were inoculated into liquid and semisolid BA-P80 media, it was shown that the semisolid media could support the growth and viability of fewer cells, especially at elevated temperatures (Figures 15 and 16).

The growth of L. hardjo at 37° C. A study was designed to determine the number of organisms that must be present

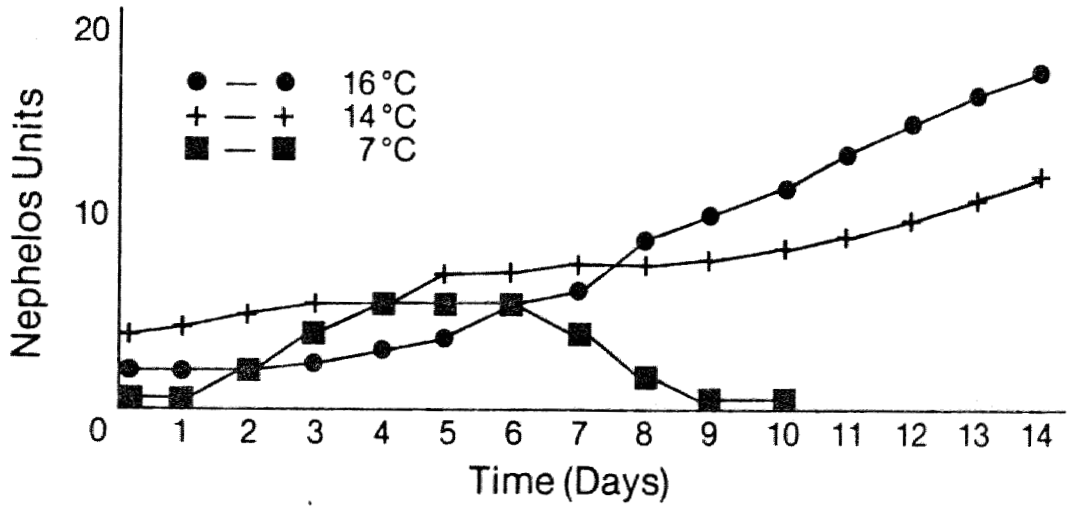

Figure 12. The growth of L. hardjo at 7°, 14°, and 16° C in BA-P80 liquid media. Each culture was inoculated with 1.0 ml of a four-day-old culture adjusted to 3.4×10^8 cells/ml.

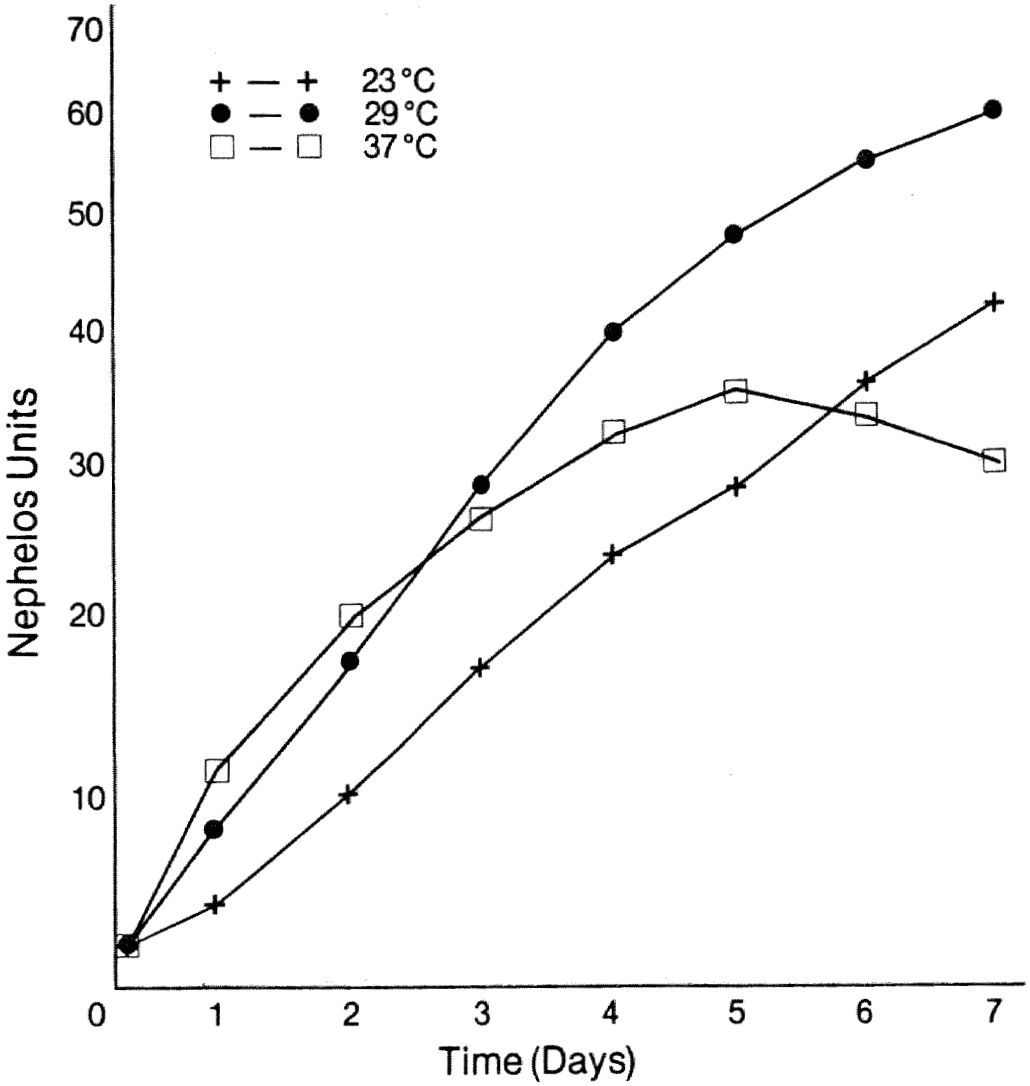

Figure 13. Growth of L. hardjo at 23°, 29°, and 37° C in liquid BA-P80 media. The cultures were inoculated with 3.4×10^8 cells.

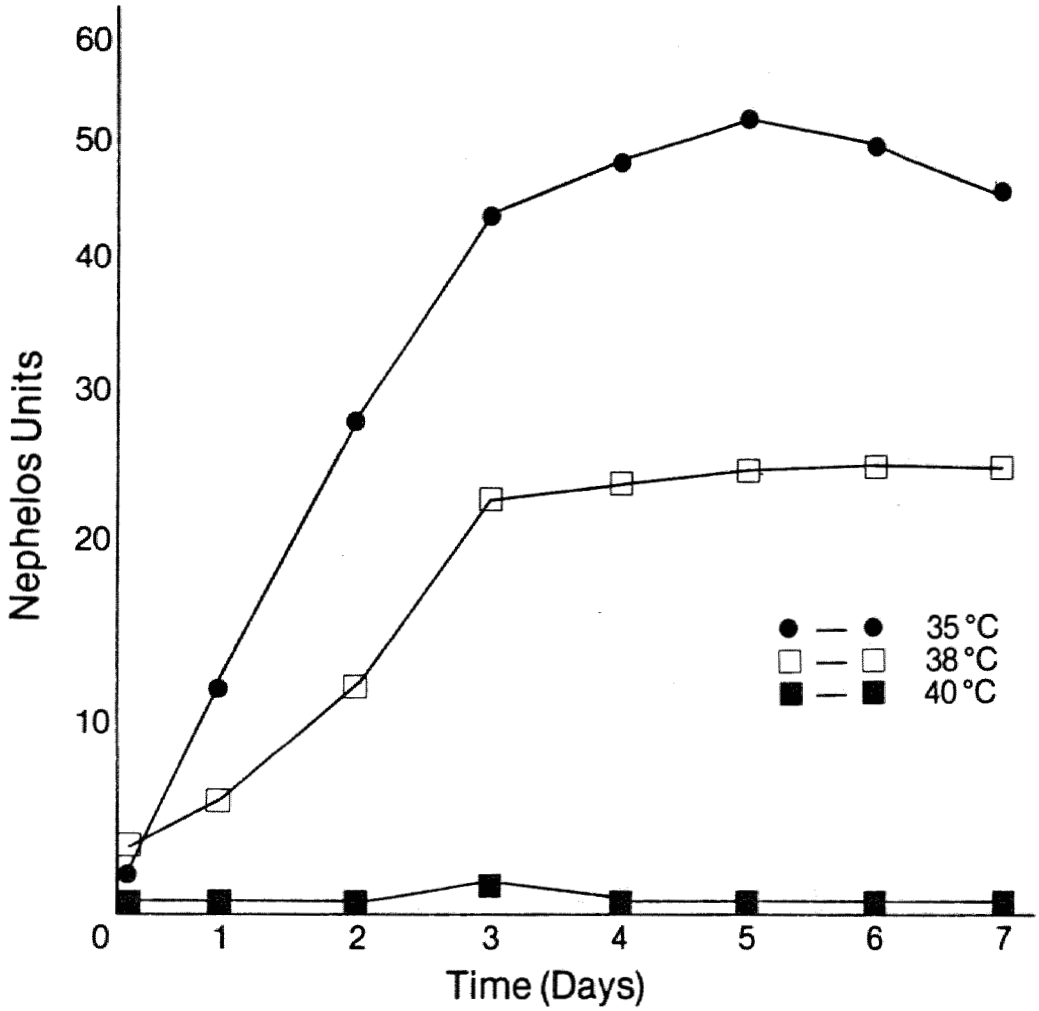

Figure 14. The growth of L. hardjo at 35°, 38° and 40° C in liquid BA-P80 media. The cultures were inoculated with 3.4×10^8 cells.

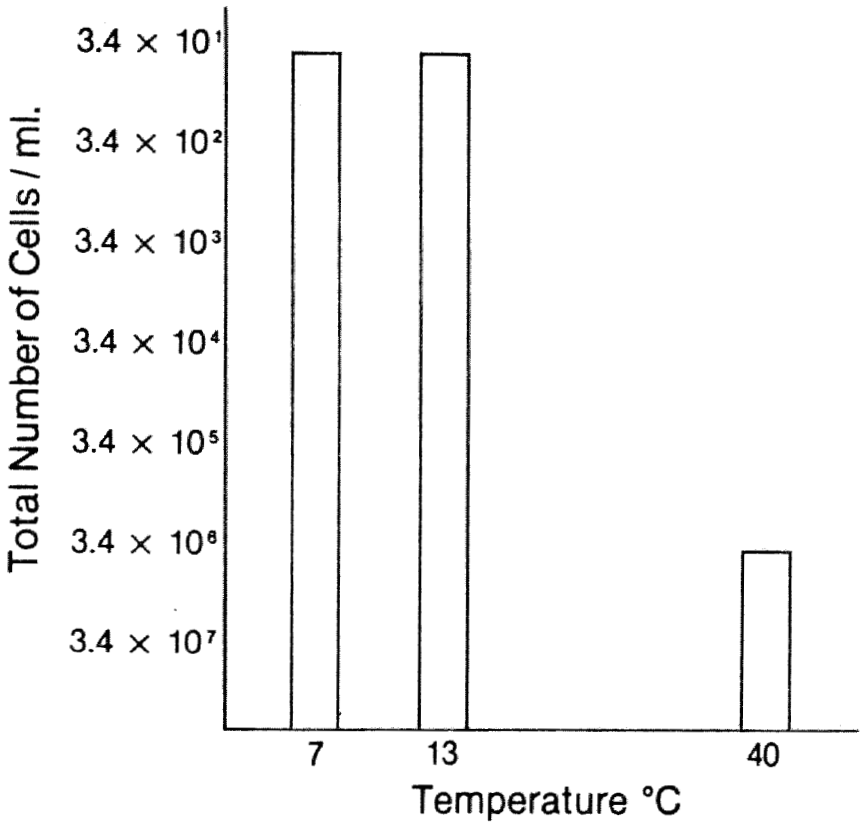

Figure 15. The growth of fewest cell numbers of L. hardjo in liquid BA-P80 media when incubated at different temperatures for 28 days.

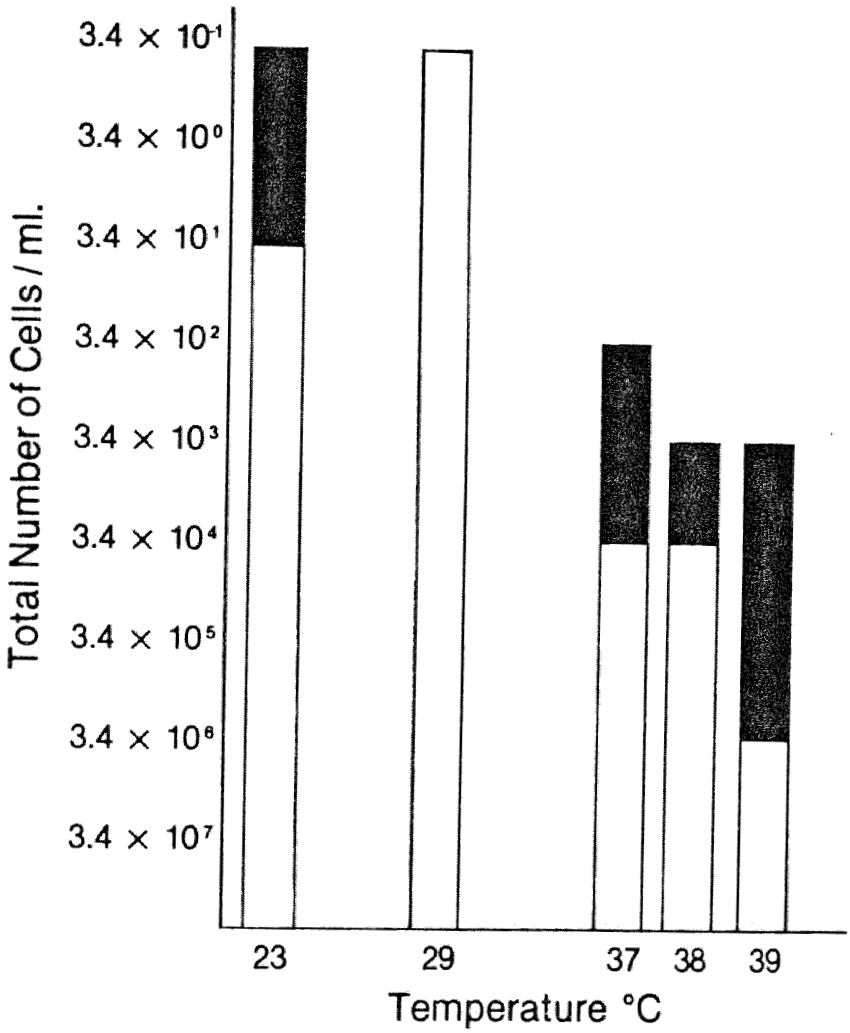

Figure 16. The growth of fewest cell numbers of leptospires when inoculated into BA-P80 semisolid and liquid media. The cultures were incubated at each of the temperatures indicated for 28 days. The shaded area represents the additional dilution of inocula that was grown in semisolid media.

to initiate continuous growth in liquid culture. The first concentration to show a continuous increase in turbidity over a three-week period was at 2.04×10^5 cells per tube (Table 15). Scale expansion techniques were used to insure that the lowest possible concentration was measured (Table 16). Viability was demonstrated at concentrations as low as 3.4×10^4 cells even though the turbidity readings were zero. The observation of viable leptospires only indicated the survival of the inoculum.

Growth of L. hardjo on solid agar plates incubated at 29° and 37° C. Two liquid cultures were incubated seven days and were used as inoculum. One culture was incubated at 29° C and the other at 37° C. A veil of growth was recorded in all four groups of plates but the enumeration of individual colonies was difficult. When the liquid inoculum was placed on BA-P80 solid plates and incubated at 29° C, four individual colonies were observed at the 10^{-5} dilution and no growth was seen at the 10^{-6} dilution. There was a hundred-fold and thousand-fold reduction in growth when the plates were incubated at 37° C. Growth of the inoculum in semisolid exceeded that recorded on plates (Table 17).

The effect of temperature on antigenicity. There was very little quantitative difference seen when L. hardjo antigens incubated at 29° and 37° C were reacted with leptospiral antisera (Tables 18 and 19). The antigen grown

Table 15

The Growth of L. hardjo in Liquid Medium Incubated at 37° C when Measured by a Nephelometer Adjusted to 52 Nephelos*

Number of cells/ml	Daily Nephelometer Readings															Viability of Last Tube
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
$3.4x10^5$	1	1	2	3	4	6	8	11	17	26	34	28	25	23	21	+
$3.06x10^5$	1	1	2	3	3	5	5	6	8	11	16	24	31	32	24	+
$2.72x10^5$	1	1	2	2	3	5	6	8	12	20	32	35	29	25	22	+
$2.38x10^5$	0	0	1	2	3	4	6	7	11	17	29	33	34	24	21	+
$2.04x10^5$	0	0	1	2	2	3	4	4	5	6	8	11	17	23	32	+
$1.70x10^5$	0	0	0	0	1	1	2	2	1	2	2	2	3	3	3	+
$1.36x10^5$	0	0	0	0	0	1	1	2	1	1	1	1	1	0	0	+
$1.02x10^5$	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	+
$6.8x10^4$	0	0	0	0	0	1	1	1	0	1	1	1	1	0	0	+
$3.4x10^4$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+

*The growth of various concentrations of L. hardjo incubated at 37° C for three weeks. Measurements were made with a Coleman 9 nephelocolorimeter using a NADC secondary standard adjusted to 52 nephelos.

Table 16

The Growth of L. hardjo in Liquid Medium Incubated at 37° C when Measured by a Nephelometer Adjusted to 104 Nephelos*

Number of cells/ml	Daily Nephelometer Readings															Viability of Last Tube
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
$3.4x10^5$	2	2	6	7	9	13	16	22	35	51	69	57	51	46	42	+
$3.06x10^5$	2	2	4	6	7	10	11	13	16	22	32	47	62	64	48	+
$2.72x10^5$	2	2	4	5	7	10	13	17	26	40	63	70	58	48	46	+
$2.38x10^5$	1	2	3	5	7	9	11	15	21	34	57	66	68	48	42	+
$2.04x10^5$	1	2	3	4	5	6	8	9	10	12	16	21	34	47	64	+
$1.70x10^5$	1	1	1	2	3	3	3	3	3	4	4	4	4	4	4	+
$1.36x10^5$	0	0	1	2	2	3	3	3	2	3	3	3	3	0	3	+
$1.02x10^5$	0	0	1	2	2	3	3	3	2	3	3	3	3	3	3	+
$6.8x10^4$	0	0	1	1	2	2	2	1	2	2	2	2	2	2	2	+
$3.4x10^4$	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	+

*The growth of various concentrations of L. hardjo incubated at 37° C for three weeks. Measurements were made with a Coleman 9 nephelocolorimeter using a NADC secondary standard adjusted to 104 nephelos.

Table 17

The Growth of L. hardjo on BA-P80 Solid Agar Plates Incubated at 29° or 37° C

	Inocula: cells per ml								
	$3.4x10^8$	$3.4x10^7$	$3.4x10^6$	$3.4x10^5$	$3.4x10^4$	$3.4x10^3$	$3.4x10^2$	$3.4x10^1$	$3.4x10^0$
29° Liquid inocula									
29° plate incubation	+	+	+	+	+	+	+	+	-
37° plate incubation	+	+	+	+	+	-	-	-	-
Semisolid control	+	+	+	+	+	+	+	+	+
37° Liquid inocula									
29° plate incubation	+	+	+	+	+	+	-	-	-
37° plate incubation	+	+	+	+	-	-	-	-	-
Semisolid control	+	+	+	+	+	+	+	-	-

Table 18

The Cross-agglutination Pattern of L. hardjo Antigen Incubated at 29° C

Serotype	Microscopic Agglutination Titer 1:10	1:100	1:1000	1:10,000	1:100,000
L. ictero M-20	-	-	-	-	-
L. naam	-	-	-	-	-
L. poi	-	-	-	-	-
L. coxi	-	-	-	-	-
L. canicola Hond Utrecht	-	-	-	-	-
L. ballum	3+	1+	-	-	-
L. pyrogenes	-	-	-	-	-
L. pomona DM_2H	-	-	-	-	-
L. gryppotyphosa	-	-	-	-	-
CDC L. Szwajizak	2+	2+	2+		
Nervig L. Szwajizak	4+	4+	4+	±	±
L. hardjo prajitno	4+	4+	3+	3+	1+
L. hardjo Downey	4+	4+	3+	3+	1+
L. hardjo BV_6	4+	4+	3+	2+	2+
L. hardjo Wolffi	4+	4+	3+	2+	1+
L. sejroe	1+	-	-	-	-

Table 19

The Cross-agglutination Pattern of L. hardjo Antigen Incubated at 37° C

Serotype	Microscopic Agglutination Titer 1:10	1:100	1:1000	1:10,000	1:100,000
L. ictero M-20	-	-	-	-	-
L. naam	-	-	-	-	-
L. poi	-	-	-	-	-
L. coxi	-	-	-	-	-
L. canicola Hond Utrecht	-	-	-	-	-
L. ballum	-	-	-	-	-
L. pyrogenes	-	-	-	-	-
L. pomona DM_2H	-	-	-	-	-
L. gryppotyphosa Moska V	-	-	-	-	-
CDC L. Szwajizak	3+	3+	1+	-	-
Nervig L. Szwajizak	4+	4+	3+	1+	-
L. hardjo prajitno	4+	4+	4+	3+	-
L. hardjo Downey	4+	4+	4+	3+	±
L. hardjo BV_6	3+	3+	3+	2+	1+
L. hardjo Wolffi	4+	4+	4+	3+	1+
L. sejroe	1+	-	-	-	-

at 29° C did show some cross reaction with the L. ballum antisera which was not seen with the 37° C grown cells. Qualitatively, the 37° C antigen appears to have formed stronger agglutination reactions. However, since most leptospiral growth is incubated at 29° C, it was adopted as the standard temperature for antigen preparation.

The growth of microscopic agglutination antigen in different Tween media. There was little difference when antigens were grown in media containing Tween 80, 60, 40, or 20 were tested with Hebdomadis serogroup antiserums. Since there was no detectable difference in end-point titers, the BA-P80 media served as the standard media for the propagation of live antigens.

Development of leptospiral agglutinins following vaccination with a heat inactivated bacterin. When hamsters were inoculated intraperitoneally with 10 micrograms of a bacterin prepared from cells incubated at 29° C there were no measurable leptospiral agglutinins produced during the first eight days post vaccination. On the tenth post inoculation day the pooled serum samples had a reciprocal agglutination titer of 40. On P.I.D. 12 the pooled serum titers were 1:80 and declined to 1:40 on post inoculation day 14.

Comparison of the efficacy of two whole cell heat inactivated bacterins. Only one of the 24 hamsters vaccinated with 10 micrograms of heat inactivated whole cells incubated

at 37° C was demonstrated to be infected. Leptospires were reisolated from the blood, liver, kidney, and brain from one hamster euthanized eight days after challenge. Kidney and liver tissues were shown to be infected at 96 hours following challenge in hamsters that were immunized with whole cell bacterin prepared with leptospires grown at 29° C.

Both bacterins provided better protection than was seen in the unvaccinated control animals. Reisolation of leptospires were made in kidney and brain tissues after 24 hours in the control group. Consistent recovery of the challenge organisms was possible from the kidney, liver, and blood after 24 hours post inoculation. L. hardjo was not recovered from the brain tissue samples until the sixth post challenge day. All urine cultures in the three treatment groups were negative.

Infectivity pattern of L. hardjo N.Z. #12 when titrated in hamsters. Leptospires were reisolated in all three tissue samples and in the urine and blood of the hamster inoculated with L. hardjo N.Z. #12 (Table 20). Two hamsters that received a theoretical challenge of three cells had infected kidneys. One culture isolation was made from liver, brain, and blood samples, also.

The pooled serum samples showed that hamsters that were challenged with at least 300 cells developed an antibody titer of 1:100, while those that were injected with 30 cells or less were less than 1:100 two weeks after challenge.

Table 20

The Infectivity Pattern of L. hardjo N.Z. 12 in Hamsters

Number of cells per animal	Number of hamsters	Number of Leptospiral Reisolations from the Tissue				
		Blood	Liver	Kidney	Urine	Brain
$3x10^6$	10	2	5	10	8	10
$3x10^5$	5	2	0	5	1	5
$3x10^4$	5	1	1	3	2	3
$3x10^3$	5	2	2	4	3	2
$3x10^2$	5	4	4	5	4	5
30	5	3	4	4	1	4
3.0	5	1	1	2	0	1
0.3	5	0	0	0	0	0

None of the kidneys revealed any evidence of severe renal lesions when examined for histopathology. Some bacteria were observed in the hamsters receiving a challenge dose of $3x10^6$ cells, but they were not positively identified as leptospires.

In an earlier experiment, it was reported that hamsters immunized with a whole cell bacterin did not develop a measurable microscopic agglutination titer until 10 days after vaccination. However, this serum with a titer of 1:80 did not protect immunized hamsters from becoming kidney infected. Only when sera with a titer of 1:1280 which occurred on day 14 was used were hamsters protected from infection (Table 21).

When serum obtained from animals that were challenged with a virulent leptospire was used a similar pattern immerged. Sera with a titer less than 1:80 provided no passive immunity against a virulent challenge introduced 24 hours later. Some protection was provided by sera with a titer of 1:1280, but all of the hamsters were demonstrated to be free of infection when administered sera with a titer of 1:2560 (Table 22).

No direct correlation could be made between the titer of the sera used for immunization and the subsequent titer of sera from the hamster two weeks later.

Table 21

Immunogenicity of the Hamster Passive Protection Test*

Reciprocal MA Titer of Antisera	Number of Hamsters	Day Sera Obtained from Vaccinates	Tissues Cultured				
			Blood	Urine	Kidney	Liver	Brain
0	1	0	-	-	+	-	+
0	1	2	-	-	+	-	-
0	1	4	-	-	+	-	+
ND	1	6	ND	ND	ND	ND	ND
0	1	8	+	-	+	+	+
80	1	10	-	-	+	-	-
160	1	12	-	-	+	-	-
1280	1	14	-	-	-	-	-

*The tissue infectivity pattern of hamsters inoculated with sera from animals previously inoculated with a heat-inactivated bacterin. All hamsters were challenged with 3.4×10^6 cells/ml of L. hardjo N.Z. #12 intraperitoneally.

ND = Animals died of nonspecific causes prior to completion of test.

Table 22

The L. hardjo Hamster Passive Protection Test*

Number of cells/ml used to produce infected sera	Reciprocal MA Titer	Number of Hamsters Inoculated	Tissues Cultured				
			Blood	Urine	Kidney	Liver	Brain
$3x10^6$	2560	2	-	-	-	-	-
$3x10^5$	2560	2	-	-	-	-	-
$3x10^4$	1280	2	-	-	1/2+	-	-
$3x10^3$	80	2	-	-	-	-	2/2+
$3x10^2$	80	2	-	-	1/2+	-	-
30	0	2	-	-	2/2+	1/2+	-
3.0	0	2	1/2+	2/2+	2/2+	-	-
0.3	0	2	NT	NT	NT	NT	NT

*The passive protection provided by the inoculation of hamsters with antisera produced against L. hardjo N.Z. #12. All hamsters were challenged with $3.4x10^6$ cells/ml of L. hardjo N.Z. #12 intraperitoneally.

NT = Hamsters died of nonspecific causes before conclusion of test.

Figures indicate number of hamsters with infected tissue/number of hamsters inoculated.

DISCUSSION

Measurement of growth. Over the last decade the use of nephelometry has become the preferred means of measuring continuous growth of leptospires. The introduction of stable titanium-dioxide standards has removed one of the biggest problems associated with nephelometry. This study illustrates the reliability of the National Animal Disease Center titanium-dioxide standards.

Secondly, the hazards associated with using a water filled cuvette with pathogenic organisms was eliminated by the development of the dry well. The dry well also removed the chance for false measurements due to an insufficient water level in the wet well. Because nephelometry closely follows Beer's law, it allows for the mathematical predictions and the manipulation of cultures previously not allowed when using light transmission instruments. This is because of the close correlation of nephelos units to cellular mass and not cell numbers.

The scale expansion method of standardizing the nephelometer was very useful when measuring slow growing organisms such as leptospires. It was also advantageous when measuring the growth of cultures containing a small number of organisms. The sensitivity of the machine can be increased or decreased by adjusting the instrument against the NADC standards.

Nutritional requirements. The laboratory adapted strain of L. hardjo used in this study was capable of growing in all four Tween products. Tween 80 which contains primarily oleic acid gave the highest measurable growth. The higher yields in media containing Tween 80 is probably due to an adaptation of this organism to the medium after many years.

The importance of the availability of long chain fatty acids was shown for Leptospira hardjo. The deletion of the polysorbate fraction resulted in decreased growth in both liquid and semisolid medium. This effect was even more evident when continuous serial subcultures were done. It would appear that the fatty acids present in the Tween is the major growth limiting fraction in BA-P80 media.

The nutritive value of the bovine serum albumin was also investigated by deleting either singularly or in combination with L-cystine, NH_4Cl, and polysorbate 80. Less growth was observed in medium containing no ammonium chloride. Even lower growth was observed in media deleted of NH_4Cl, polysorbate and L-cystine in combination or in media without just a polysorbate component. The presence of bovine serum albumin alone could not supply sufficient essential nutrients that would promote the growth of L. hardjo to a density equal to complete BA-P80 media. The absence of either vitamin B_1 or B_{12} did not greatly reduce the growth of the organism after one subculture from complete BA-P80 medium.

When the organisms were washed in diluent, the growth levels were much lower.

It has been suggested that leptospires are capable of storing vitamins B_1 and B_{12} and as these reserves are used up, the total measurable growth diminishes. Previous attempts to demonstrate a requirement for vitamin B_{12} has been unsuccessful. The addition of B_{12} to a medium containing serum failed to increase the growth of leptospires. This failure was probably due to the amount of vitamin B_{12} already present in the animal serum supplement (Gregory and Holdsworth 1959). Roberts (1972) reported that a high liver hamster passage isolate of L. icterohaemmorrhagiae had the ability to store vitamin B_{12} until it had been passed seven times in liquid medium containing no additional B_{12}. However, the cells were not washed prior to each subculture. A carryover of vitamin B_{12} may have been responsible instead of endogenous storage by the organism.

The requirement of thiamine (vitamin B_1) has been previously described. Three successive subcultures of L. Hardjoprajitno were made in medium containing no vitamin B_1. No attempts were made to define how long continuous subcultures could be done. When vitamin B_1 or B_{12} deficient semisolid media were inoculated with one ml from serial ten-fold dilutions, macroscopic growth was observed only one and two dilutions lower than in complete media.

No requirement for L-cystine could be demonstrated for L. hardjo. No significant decrease in turbidity or antigenicity was observed when using various concentrations of inoculum in medium containing no L-cystine. Leaving L-cystine out of the BA-P80 media would not hinder the growth of L. hardjo culture and it would eliminate a time consuming filtration step.

The growth of L. hardjo in gamma irradiated albumin. The use of bovine serum albumin powder in BA-P80 media requires a series of filtrations to sterilize rehydrated albumin. The powder does not go into solution rapidly and easily clogs membrane filters with a porosity less than 0.45 microns. Sterilization by irradiation is quicker and allows larger batches of albumin to be sterilized at one time than using standard filtration techniques. The use of irradiated bovine serum albumin was not shown to be detrimental to the growth of laboratory adapted strain in L. hardjo except when albumin was treated with dosages greater than 2.5 megarads.

Currently, 2.5 megarads of radiation is generally accepted as sufficient for the sterilization of absorbable surgical sutures. Dosages in excess of 4.5 megarads are needed for complete inactivation of spore-forming organisms such as Clostridium botulinum (Russell 1965). Therefore, care must be taken to insure that enough radiation is used to insure sterility while not jeopardizing the nutritional

qualities of the albumin.

The importance of bovine serum albumin in BA-P80 medium. Bovine serum albumin in a concentration of 1% in a phosphate buffer has been reported to maintain viability of leptospires (Roberts 1972). It has also served as a good transport medium for blood and urine specimens from infected animals. Roberts' work with L. icterohaemorrhagiae HLVP demonstrated viable organisms in a solution of 1% or 1.5% BSA, 0.005% M phosphate buffer, and distilled water.

This study reports the progressive growth of an avirulent L. hardjo in BA-P80 media containing no albumin. Although the turbidity was less than in the control media, five subcultures at seven day intervals in media containing 0% BSA were possible. This phenomenon may represent a carryover of micronutrients from previous passages in complete bovine serum albumin P-80 media. Bacterial growth equal to the growth measured in the control medium was seen when the albumin concentration was 2.0%. When the albumin concentration was increased to 15.0% or more, the maximum turbidity measured over the one week period was less than the control. By the fifth continuous subculture, a 1.0% concentration of albumin was needed for the turbidity of the culture to be equal to the control. Further subcultures away from the original culture would be necessary before any conclusions could be drawn from this study.

The effect of temperature on growth. The growth studies were designed to establish the range of temperatures that would support viable organisms and continuous progressive growth of L. hardjo. Previous investigators have reported that successful laboratory propagation is best achieved at temperatures between 23° and 31.5° C (Ellinghausen 1971).

The optimal incubation temperature where daily increases in turbidity reading were measured occurred at temperatures between 23° and 31.5° C. However, the lower the temperature, the longer the period of time necessary to reach the maximum turbidity. The lag phase in the growth curve was reduced when the leptospires were incubated at temperatures between 35° and 37° C. However, the maximum turbidity readings were always less than the maximum values measured of cultures incubated at 29° C. The fact that leptospires can be grown at or near room temperature makes studies of this organism possible in those laboratories lacking sophisticated incubation devices.

It was also interesting to find that the growth of L. hardjo could be accomplished by using semisolid BA-P80 medium at elevated temperatures that had previously proved lethal when the organisms were grown in liquid BA-P80 medium. Perhaps this is due to some contaminating micronutrient that protects the leptospire. No attempt was made to measure the actual temperature in the tubes containing

semisolid media. It is possible that the actual temperature in the culture was lower than the temperature of the water in the bath. It is possible that the ability of the BA-P80 semisolid media to support growth at elevated temperatures may have resulted from some sort of phenotypic selection from the general population which resulted in a temperature sensitive mutant. Similar results were observed in studies designed to allow the enumeration of colonies on BA-P80 solid plating medium. The plates incubated at 37° C did not support the growth of an inoculum containing a few cells. However, the same inoculum was able to grow at 37° C in a semisolid BA-P80 medium incubated at 37° C.

Ellinghausen (1971) has previously published information on the ability of 27 strains of L. pomona and four strains of L. grippotyphosa to grow at a temperature of 37° C. He found that there was a great deal of variation in the ability of organisms within the same strain to grow at a temperature of 37° C. In this study L. hardjo Hardjoprajitno was shown to be capable of growing in liquid BA-P80 media when incubated at 37° C only when inoculated with a large concentration of organisms.

Perhaps this strain of leptospire has mutated during the many years that it has been maintained in the laboratory and now possesses enzymes which are capable of functioning at elevated temperatures. It may also be possible that a small number of organisms that could replicate at

this temperature were "naturally selected" and began replicating progressively.

The effect of temperature on antigenicity. There appeared to be no measurable difference between the cross agglutination reaction using antigens grown at either 29° or 37° C.

When seven members of the Hebdomadis serogroup were grown in media containing Tween 20, 40, 60, or 80, differences were seen in antigens using Tweens 40 and 20. Several authors (Stalheim and Wilson 1964a; Semenova and Soleshenko 1967; Ellis 1970) reported alterations in antigenicity based upon the nutritional environment. Perhaps the fatty acids contained in Tweens 40 and 20 do not provide the necessary nutrients that allowed the proper synthesis of the antigenic determinants measured by the microscopic agglutination test.

Development of leptospiral agglutinins following vaccination with a heat-inactivated bacterin. The exposure of whole cell cultures of L. hardjo to a temperature 65° C for one hour did not destroy the antigenicity of the organisms as measured by the microscopic agglutination test. The leptospirosis-susceptible hamsters responded to the bacterin in a very predictable manner consisting of a lag period of several days at which time no measurable immune response was observed. This was followed by a short-lived increase in antibody followed by a period of decline. A study using

various dosages of antigen would provide some idea of how quickly the immune response followed the injection of the bacterin. Perhaps the 10 micrograms of antigenic material was too little to stimulate a maximum response. Likewise, the length of the immunity may also have been correlated to the dosage of bacterin administered. More serum samples taken over a longer period of time would have been helpful to determine the length of time that the immunity persisted following immunization.

Efficacy of two heat-inactivated leptospiral bacterins. The vaccinated challenge experiment showed heat inactivation to be a simple, inexpensive and practical method of producing efficacious bacterins. Nervig et al. (1977) reported similar findings using a heat-inactivated Leptospira interrogans serotype szwajizak bacterin in hamsters. Presently, most bacterins used for the prevention of leptospirosis are produced using fermentation methods. The bulk fluids are harvested and chemical inactivating agents are added to the cultures. However, the use of heat-inactivation would be easily accomplished by raising the temperature of the fermentor to a predetermined temperature for the necessary time needed to kill all viable cells. This procedure is simpler, and can be done with less chance of contaminating the bulk cultures. It also eliminated the need for the addition of any chemicals which may be harmful to the recipient animals.

The bacterin using leptospires grown at a temperature of 37° C was more efficacious than the bacterin using cells grown at 29° C. Perhaps the use of the higher incubation temperature stimulated the production of more antigen, or an antigenic determinant not present at the lower temperatures. The incubation at 37° C may also cause some type of phenotypic expression or selection of a more antigenic population. Further studies would need to be done in order to explain this phenomenon.

Titration of virulent L. hardjo challenge. The viscertrophism of the virulent L. hardjo NZ 12 organism appeared consistent with the infection pattern of other leptospires. During the first 24 hours after challenge, the organisms were reisolated from the liver and kidney tissues. These are probably the first organs to become infected, because of the volume of blood that passes through them. Localization in these organs may also be due to their favorable nutritional environment. Pearce and Lowrie (1972) demonstrated that the lipids on some cell surfaces were susceptible to breakdown by leptospires. The inability to recover leptospires from the urine of infected hamsters may have been due to the short duration of the test. Perhaps if samples had been obtained several weeks after the injection of the challenge it would have allowed sufficient time for the organisms to establish persistent infection in the kidney tissues.

Even though reisolation of viable leptospires was possible from kidney tissue homogenate, there was no evidence of renal lesions reported after histopathological examination. Some bacteria were observed in the kidneys of hamsters that were inoculated with 3×10^6 cells, but they were not positively identified as leptospires (Larson, 1977, personal communication).

Hamster passive protection test. Sera from hamsters with a reciprocal microscopic agglutination titer of 1,280 provided immunity against challenge when inoculated into a susceptible hamster. Sera with titers greater than 80 appeared to prevent all but infection of the renal tissues. However, these findings must be considered preliminary due to the small number of animals included in each test group.

CONCLUSIONS

The routine use of the National Animal Disease Center nephelometer standards and the aluminum dry well demonstrated that they were easy to use and provided results that were reproducible. The 50.5 nephelos rated standard was found to be the most useful. When the nephelometer was adjusted to a turbidity of 25 nephelos against the standard, it could be used to measure dense cultures. When the instrument was adjusted to twice the value of the 50.5 nephelos standard, the growth of dilute or slow growing cultures was easily measured.

A laboratory adapted avirulent strain of L. hardjo Hardjoprajitno was tested for growth and viability in sixteen substituted and deleted forms of liquid bovine albumin polysorbate 80 (BA-P80) medium. The original Ellinghausen-McCollough formulation of BA-P80 was used as the standard for most of the research described in this study.

The absence of any of the polysorbate fractions reduced the measurable growth. When polysorbate 80, NH_4Cl, and L-cystine were all deleted, growth was minimal. These two findings confirm the requirement of L. hardjo for nitrogen and long chain fatty acids.

If either vitamin B_1 or B_{12} was deleted from the medium, the amount of growth decreased. Each successive subculture in the absence of vitamins resulted in lower nephelometer readings. Only two subcultures were necessary before L. hardjo failed to grow in media without vitamin B_{12}. The deletion of either of the two vitamins also reduced the ability of the media to support the growth of minimal cell numbers.

The role of the agar used in preparing semisolid BA-P80 revealed that there was variation in the grades and lots tested. No attempt was made to determine why the variations were observed. Subsequent studies showed that the quality of the bovine albumin used played a greater role than the quality of the agar when growing minimal cell numbers.

Eighteen different albumin samples were tested for the

ability to stimulate maximum growth of L. hardjo in liquid BA-P80 media. Equine and porcine albumin did not possess good growth supporting ability. Albumins that had been adjusted to pH 7.0 to 7.8 did not differ from one another.

Bovine albumin fraction V powder that had been sterilized with gamma irradiation was tested. The sample that had been given 2.5 megarads did not grow cultures as turbid as the untreated control albumin. This may have been a result of destruction of some micronutrients bound by the albumin or the protein structure of the albumin molecules.

The optimal incubation temperature was between 29 and 31.5° C. L. hardjo was capable of growth at 37° C but at reduced levels. Growth was possible at temperatures above 37° C only in BA-P80 semisolid media, especially when only a few cells were used as inocula.

No difference was observed in the antigenicity of organisms grown at 29° or 37° C. Neither was there any difference in the antigenicity of cells grown in the presence of the four Tweens.

The heat-inactivated whole cell bacterin prepared with leptospires incubated at 37° C was more efficacious than a bacterin using cells incubated at 29° C.

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